USING CRISPR/CAS9 TO MODIFY THE GENOME OF CATTLE

BY

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THESIS

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Abstract

Genetically modifying animals is a tool that can be used to increase livestock production. The gene editing technology CRISPR has expanded the possibilities of gene editing. The Cas9 nuclease creates a double strand break which can be repaired by non-homologous end joining (NHEJ) or homology directed repair (HDR). The goal of this experiment was to create a single nucleotide change using the CRISPR/Cas9 system in combination with a single strand oligo nucleotide (ssODN). The single nucleotide that was targeted occurred naturally in Holstein cattle, and is associated with increase milk production. There are multiple factors involved in the Holstein cow's ability to produce large amounts of milk. A mutation in the alpha lactalbumin sequence (α-LA) is one of these factors. The α-LA gene sequence in some Holstein cattle contain an adenine the at (+15) position which corresponds to transcriptional start point of α-LA (+1), while other breeds have a guanine at that position. The adenine in the +15 position of the α-LA gene has been associated with increased milk production. MAC-T cells and Angus fetal fibroblasts were transfected with a Cas9 plasmid, pSpCas9(BB)-2A-GFP, and a ssODN to insert the desired mutation. Using the CRISPR/Cas9 system we were able to create a double strand break resulting in indels and deletions at the (+15) site in MAC-T cells, but we were not successful in creating a single nucleotide change. However, we did see a single nucleotide change in Angus fetal fibroblasts using CRISPRs and ssODN. Following the success of inserting the mutation into a cell line we attempted to create an embryo containing the single nucleotide change using sperm-mediated gene transfer (SMGT). Naked DNA binds naturally to sperm, and can be used to produce transgenic offspring in pigs (1) and cattle (2). In this experiment, we analyzed methods to select thawed bovine sperm, and evaluated the binding of exogenous DNA to those sperm. Liposome preparation was done using a cationic lipid, 3-(trimethyl ammonium iodide) 1,2 dimystryl-propanediate (TAID) and a neutral lipid, L-α Dioleoyl phosphatidyl-

ethanolamine (DOPE) prepared according to given protocol (3). Percoll or swim-up methods were used to select sperm after thawing (4), followed by incubation (1h or 3h) with the liposome-DNA complexes (3). We used enhanced green fluorescent protein (eGFP) in combination with the liposomes as a marker for exogenous DNA binding. Five treatments per selection method were analyzed: 1) no incubation, no liposomes and no DNA, 2) incubation with no liposomes and no DNA, 3) incubation with liposomes and no DNA, 4) incubation with liposomes and 1 ng of DNA and 5) incubation with liposomes and 10 ng of DNA. Once the liposomes had been complexed with DNA they were incubated with sperm for one or three hours before IVF. The CASA results for total motility and rapid motility were significantly different from the control ($P<0.01$) between the control and the other treatments in the Percoll group as opposed to swim-up. These results confirm that the sperm selected with swim-up has less effect on sperm motility than Percoll. Real time PCR was able to detect plasmid GFP DNA in the DNA of sperm samples and pictures taken of the sperm using the Spatial Light Interference Microscopy (SLIM) confirmed the presence of liposomes on the sperm head and tail. SMGT was then used with IVF to deliver two plasmids containing GFP encoding region under the control of CMV promoter; pIRES2-EGFP and pSpCas9(BB)-2A-GFP for gene transfer. The blastocyst rate was low and ranged from 0 to 20%. Although we did show that plasmid DNA was present on the sperm, we were not able to detect any positive GFP 8 day embryos using SMGT.

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Introduction

In 2050, the expected size of the worldwide human population is ~9 billion, the demand for food will increase and the demand for milk will increase along with it (5,6). As countries develop and people rise above poverty level the demand for meat and milk increase. The main increase in milk demand will occur in third world countries, by 2020 it is estimated that the population will consume 177 million metric tons more milk than in 1996/1997 (7). Since 1950 milk production has more than doubled due to artificial insemination (AI). Although some of the increase is due to advancements in feed and management the majority of increased production has come from the ability to select animals with higher genetic merit (8). Genetically superior traits can be utilized even further by using biological tools to insert these traits into animals to provide food for the demand of the growing population. In the United States, Holstein cattle are the leading dairy breed for milk production and produce an average of 24,291 pounds of milk per year. One of the genes involved in their ability to produce large quantities of milk is linked to a single nucleotide change in the 5' promoter region of α-lactalbumin (α-LA) (9). In theory, this mutation could be inserted into breeds that produce less milk to increase production using gene transfer.

One of the more recent gene editing technologies is clustered regularly interspaced short palindromic repeats known as CRISPRs. The Cas9 nuclease can be used to make site specific cuts in the DNA, these cuts are then repaired by non-homologous end joining (NHEJ) or homology directed repair (HDR). NHEJ may repair the sequence or it may lead to the creation of indels, insertions or deletions, that may lead to a frame shift or premature stop codons which will knock-out gene expression. A repair template can be used to insert mutations through the process of HDR. This system is able to make large insertions or a single nucleotide change. The

CRISRP/Cas9 system can be used to genetically manipulate cultured cell lines as well as embryos and be delivered in a variety of ways (10). Combining new gene editing technologies with gene transfer methods opens new possibilities for feeding the growing population.

Chapter 1

Literature Review

1.1 The Production of Milk

The mammary gland differs widely from other organs; it is characterized by being highly differentiated and one of the most metabolically active organs in the body. It also stands out as an organ that does not offer the animal itself much support but puts a large demand on the animals body when it is functioning at its greatest potential (11). This demand occur largely after birth unlike other tissues that mainly develop before parturition, mammary development predominantly occurs postnatally (12). When lactation begins the entire animal undergoes great change, there is a redistribution of the blood supply, an increase in metabolic rate, and an extreme increase in nutrient demands (11). If the animal cannot meet these demands the result can be metabolic disorders (11). These changes show the importance of lactation and what the body will go through in order to accommodate the demands of the mammary gland.

1.2 General Cellular Components

There are many components that are needed for the mammary gland to fully function. Starting with the nucleus, at the beginning of lactation, there is an increase in nucleic acid content, and thus an increased amount of RNA, eventually resulting in an increase in protein production (11). The increase of proteins at parturition are not the same as the ones that are secreted in the milk but are associated with cell components. These proteins have a more regulatory effect on metabolism (13). The cytosol also plays a role in milk formation, it contains enzymes that catalyze reactions such as generating intermediates and cofactors that are needed for milk synthesis. As lactation begins epithelial cells require an increase in energy, this results in an increase of mitochondria seen in epithelial cells. Epithelial cells need energy for massive

and active transport as well as for secretory activity. In cattle, these mitochondria take on various forms and are widely distributed, this is attributed to the cell crowding with organelles (13). Now that the cell has energy and has started producing enzymes, milk synthesis is set up to begin production. The final piece is the microsomal fraction which contains the Golgi apparatus, endoplasmic reticulum, and lateral and basal cell membranes, along with some other minor organelles (14). These organelles are in charge of taking constitutive parts such as amino acids, glucose or fatty acids and assembling them into a finished product like proteins and lactose. These products are then secreted from the cell and into the milk.

1.3 Metabolism

As explained previously, the cellular components involved in milk production require the production of energy to function properly. Dietary nutrients play a large role in in milk synthesis. For example, non-ruminants receive their energy from glucose which is derived from dietary carbohydrates. In contrast, ruminants use microbial fermentation products (acetate, propionate, and butyrate) as energy and carbon sources for tissue metabolism (11). Ruminants tend to restrict the use of glucose in synthetic and oxidative reactions leading to the use of other substrates. One of these substrates is acetate, which is the primary substance during fatty acid synthesis and is derived from fermentation of carbohydrates in the rumen. The cow has utilized its energy source by excluding glucose as a carbon source from fatty acid synthesis, thus pushing reliance on acetate which is highly produced in ruminants (11). Ruminants are unique in their ability to use microorganisms that live in the rumen and produce a higher amount of acetate, allowing the breakdown of substrates which would otherwise have little nutritional value (15). Another source of energy comes from simple metabolites derived from the blood. Some common substrates are

glucose, acetate, ß- hydroxybutyrate, amino acids and fatty acids, which all play a role in establishing milk composition (11).

Glucose is a key player in metabolism of the mammary gland. In a study done where glucose was restricted by administering insulin, a decrease in milk volume was seen (16). Glucose is significant because when other metabolites are altered, such as acetate or amino acids, the milk volume does not greatly vary but leads to a change in the amount of fat and protein, unlike glucose which can alter the volume (17). Glucose enters the mammary secretory cell and then one glucose is used without modification while the other is converted to UDP-glucose and then to UDP-galactose; both of these are used in lactose synthesis (11). Another portion is phosphorylated to glucose-6-phosphotase by hexokinase. Lactose synthesis requires the combination of glucose and galactose and the galactose comes from glucose-6-P (18). It is estimated that 50-60% of the glucose that becomes glucose-6-phosphotase is then converted to galactose and used for production of lactose. The remaining glucose-6-phosphotase is metabolized by the pentose phosphate and Embden-Meyerhof pathway, which primary function is to generate NADPH for fatty acid synthesis. The Embden-Meyerhof Pathway is also used for synthesis of glyceride-glycerol, which is then combined with other pathways and assists in the production of carbon for the synthesis of fatty acids, amino acids, and the production of energy. There are other pathways and substrates involved in production and utilization of energy but this is the extent of the discussion for basic mammary metabolism.

1.4 Overview of α-Lactalbumin

α-Lactalbumin (α-LA) is a major whey protein that plays a key role in bovine milk production (19). The increased expression of α-LA is associated with the increase of milk

production, which is primarily due to the role it plays in the production of lactose (18). Dairy cows produce ~1.5 g/L of α-LA and ~5% lactose (20). Lactose is the predominant sugar of most milks and makes up 2-7% of milk in most mammalian species. Lactose and the ions Na+, K+, and CL- are primarily responsible for regulating the osmotic pressure of milk and contributes to determining the aqueous phase of milk acting osmotically to draw in water (21).

Two proteins were found to be involved with enzymatic activity that catalyze the biosynthesis of lactose, β-1,4-galactosyltransferase and α-lactalbumin. The primary role of β-1,4-galactosyltransferase is to transfer galactose to the carbohydrate side chain of glycoproteins (18). α-Lactalbumin (α-LA) is synthesized on the ribosomes of the rough endoplasmic reticulum and then passes into the tubules and vesicles of the Golgi where it can then interact with β-1,4-galactosyltransferase to synthesize lactose (22). The production of lactose is dependent on the amount of α-LA present. When α-LA moves through the Golgi binds to the β-1,4-galactosyltransferase, UDP galactose, and glucose to form lactose. The normal function of β-1,4-galactosyltransferase is to glycosylate proteins and glycolipids. However, when α-LA binds to β-1,4- galactosyltransferase it shifts the function to increase production of lactose (22). Since α-LA exists in milk along with lactose, other sugars and proteins, it has been theorized that α-LA also plays a role in the secretion of lactose into milk (22).

1.5 Hormonal Control of α-LA

The production of α-lactalbumin is controlled by hormonal regulation in the mammary tissue. When an animal becomes pregnant, hormones are released to prepare the body to carry the offspring and then eventually lactate. In order to better understand the hormonal regulation of α-LA in cattle, Goodman et al. studied secretion of α-LA in bovine mammary tissue that was

cultured *in vitro* (23). Tissue was removed surgically from the caudal aspect of a front mammary quarter from 16 multiparous non-lactating cows and then put into culture medium. They found that prolactin (PRL) is one of the primary modulators of α-LA in bovine mammary tissue (23). Similarly, estradiol plays a role in synthesizing α-LA as well as promoting growth in the mammary tissue. Estradiol also increases the amount of PRL- binding sites, allowing PRL to have an increased effect on α-LA production. On the other hand, progesterone has the opposite action and inhibits the effects of PRL. It has been suggested that one of the factors that initiates lactogenesis is the decrease of progesterone (23). Cortisol is another hormone that enhances the effects of PRL. Cortisol is required for mammary secretory tissue differentiation; it stimulates the formation of rough endoplasmic reticulum and the Golgi apparatus within the mammary secretory cell during pregnancy (23). Although cortisol does not have a direct effect on α-LA production it does enhance the effects of PRL on lactogenesis (23).

1.6 Genetic Control of α-LA

The mature α-LA sequence contains 123 amino acids and a signal peptide that is composed of 19 amino acids. Together this makes four exons that are transcribed into 724 base pairs on chromosome 5 (24-27). In 1993, Bleck and Bremel sequenced the α-LA coding gene in different breeds of cattle and compared the 5' flanking region and the protein coding region to search for point mutations. Polymorphisms were found at +15, +21, and +54 relative to the mRNA transcription start point. The mutation at position (+54) was a silent mutation, located in the SP-coding region of the gene, and was therefore disregarded. Polymerase chain reaction (PCR) was used to analyze the frequency of the (+15) polymorphism because it was easier to detect. Randomly selected cattle (n=501) composed of seven different breeds were screened for

the (+15) mutation, and it was not seen in any breed except Holsteins, which contained the mutation at a frequency of 32% (9). Further studies were performed to determine if this mutation was correlated with milk production. They found that α-LA concentrations were positively correlated with milk protein, milk fat, and lactose concentration (19). This study only looked at the effect of α-LA (+15) in the Holstein population. Four- hundred and eighty (480) Holsteins were used in this study, the polymorphisms were labeled as α-LA (+15) A and α-LA (+15) B. α-LA A was used to label the variants containing an adenine at the (+15) position, α-LA B was used for variants contacting guanine. They saw that AA animals had a higher potential transmitting ability (PTA) for milk ($P < 0.01$), protein yield ($P < 0.05$), fat dollars ($P < 0.1$) and fat yield ($P < 0.05$). The BB animals had higher PTA for protein ($P < 0.01$) and fat percentages ($P < 0.05$), and the PTAs for AB were in between AA and BB. Bleck and Bremel also looked at the α-LA (+15) region in caprine, ovine, and human, and saw that guanine was preserved across species except in bovine variant A (19). Similarly, when comparing Holstein breed to the Nelore breed, Holsteins were the only breed that contained allele A on position (+15) (28). Based on these results this mutation is strongly associated with increase in milk production and could be utilized in gene editing scenarios to increase milk production in other cattle breeds.

It has been theorized that this mutation leads to increased milk production by increasing the affinity for binding Nuclear Factor 1 (NF1) gene. The mammary gland is unique in that it predominantly develops postnatally. This leads to the requirement of both ubiquitous and cell-type specific promoter-DNA interactions, these interactions may involve the NF1 family (12). NF1 was originally purified from HeLa cell nuclear extracts and was shown to be essential for *in vitro* replication of adenovirus DNA (29). Further studies showed that NF1 leads to replication by recruiting viral replication proteins to the replication origin and that it targets the DNA

binding domain of the protein (30,31). NF1 initiates transcription by binding to DNA containing the sequence TGG(C/A)N_5GCCAA (32-34). When the role of binding proteins was examined in the 5' flanking region of α-LA in rats, they found two binding sites that were highly conserved (35). By using a DNAase I-footprinting assay to analyze binding proteins researchers found the sequence TTGGCAG (35). This site aligns with the NF1 binding sequence as stated previously. It is theorized that since binding activity is similar to that of the NF1 protein binding, proteins that bind to α-LA 5' flanking region may be part of the NF1 family. The mutation found by Bleck and Bremel in cattle that is associated with increase milk production results in a sequence change of TGGGGGGTA to TGAGGGGTA (9). This mutation may increase the binding affinity for binding factors associated with the NF1 family, thus resulting in increased production of α-LA.

1.7 The Importance of Milk

Cow milk has been consumed worldwide for thousands of years and has been described as "the nearly perfect food" for humans. It is a complex biological fluid composed of calcium, magnesium, selenium, riboflavin, vitamin B_{12} and vitamin B_5, as well as other important nutrients (36,37). Most countries suggest one serving of milk per day and in the past 50 years' consumption of milk in developing countries has doubled. In the past 40 years this demand has been met by increasing milk production in cows (38). In the US alone between 1957 and 2007, the average milk production increased by 5,997 kg and 56% of this increase was due to increased genetic merit (39). The increase in milk production was aided by the introduction of artificial insemination (AI) technologies. AI provided the ability for wide spread of high quality bull semen, which was determined to be of higher merit through the use of progeny testing. Progeny

testing looks at the value of the breeding individual based on the progeny's production value. The advent of quantitative genetics, which came about in the 20th century, used statistical theory to analyze phenotypes by identifying variations at loci, leading to the discovery of small changes that made large impacts (40). Not only has selection increased production, it has also lead to a decrease in the environmental impact. Today, in order to produce a gallon of milk, it requires 65% less water and 90% less land than it did in 1944 (41).

Even with the increase in milk production there is still a great lack of sufficient dietary energy in developing countries. From 2010 to 2012, 870 million people died because they did not have enough food and of those 852 million people were in developing countries. Malnutrition in these countries can result in stunted growth, in 2011 26% of all children in the world suffered from stunting, and 90% of those children were in Africa and South Asia (36). It is estimated that by 2050 the demand for meat and milk will more than double again in developing countries (42). Currently improved genetic traits have been achieved by crossbreeding to mix desired traits and the selection, which utilizes genetic variation to select the traits with greater advantage. Genetic engineering is a another way in which production can be improved, this method allows for the generation of new and diverse genetic traits (43).

1.8 Transgenic Animals

Before transgenic animals, artificial selection was used to increase animal production by breeding for desired agricultural traits. Gene editing technologies have now advanced the ability to enhance production of agricultural animals. In 1971, the first transgenic animal was made using sperm-mediated gene transfer in rabbits with viral DNA (44). Although it was not until 1976 when new born mice and mouse embryos were infected with viral DNA, in both cases the animals took up the transgene and were able to pass the disease phenotype on to their offspring

(45). Transgenic animals can be used to increase livestock production, create medical models, decrease environmental impact, gain knew knowledge and improve animal welfare, among other things (46-48). Genetic engineering usually involves “gain of function” or “loss of function.” Gain of function is the insertion of DNA, or the removal of an inhibitor to give or increase a function that the animal did not previously have, while loss of function alters a DNA sequence in a way in which it can no longer be properly expressed and thus results in a loss of function. In the past few years TALENs and CRISPRs have allowed for directed gene edits, these methods can be designed to target more specific sites on the genome and create a double stranded break (49). Transgenic animals provide an opportunity to rapidly make genomic changes without years of crossbreeding.

1.9 Gene Transfer Methods

Gene transfer is often used to alter the genetics of animals to increase their production. There are multiple ways to transfer genetic material. One of the most common techniques is microinjection, this method injects foreign DNA into the pronucleus of a fertilized egg and works very efficiently in mice (50-54). While this method is very effective in mice, it is not as efficient in livestock species. This has led to the use of other methods such as sperm-mediated gene transfer (SMGT) or somatic cell nuclear transfer (SCNT). SMGT does not require as much skill as nuclear transfer, but there are varying efficiencies and results are often difficult to repeat (55). Nuclear transfer uses embryonic or somatic cells, that have been edited, to make clones with the desired gene edits and have a greater success rate than SMGT but requires greater expertise (56,57). Other methods do exist but these are the main three that will be the focus of this discussion.

While SMGT was the first method used to create a transgenic animal, microinjection has been shown to work very well in mice (58). Transgenic animals are animals that have had DNA inserted and then stably integrated into their chromosomes. Microinjection has been widely used and as stated previously is most successful in mice (50-54). This method is used in mice by removing the embryo from the recently fertilized animal. Then under a microscope, micromanipulators are used to grasp the embryo. Once the embryo is immobilized foreign DNA can be inserted into the embryo. Following the injection, the embryo is transferred into the female and gestation can proceed as normal. The advantages of using this system is that a high rate of gene transfer is seen (59). The disadvantages of this technique are: 1) the method cannot be used with embryos of later developmental stages; 2) screening of the host chromosomal insertion site is difficult because the DNA integrates as multiple copies, which are configured in a tandem head-to-tail manner or array and 3) livestock species have a much lower frequency of integration of foreign DNA into their chromosomes, which makes production expensive due to need for large number of transfers.

Nuclear transfer has been more efficient in generating transgenic livestock and is now a commonly used method. The first animal created using this method was “Dolly” the sheep and since then been used to generate sheep, cattle, mice, goats and pigs (60). The genetic material is first removed from the cytoplasm, this step is call enucleation, which then leaves just the cytoplasm. After enucleation of the oocyte, a donor nucleus, or karyoplast, is injected into the perivitelline space of the enucleated oocyte. Once the oocyte is enucleated, the oocyte and donor cell are fused by electrofusion. This step varies depending on the species. Following fusion, the oocyte is activated by chemical means or electric stimulation. If the activation is successful development will proceed to the blastocyst stage and transferred to a recipient. Nuclear transfer

can be done with embryonic stem cells (61), somatic cells, or more recently induced pluripotent stem cells (iPSC). While nuclear transfer has been used and demonstrated in multiple species, the success rate can be very low (62). Embryonic stem cell (61) and nuclear transfer technologies are still experimental, and a considerable amount of variables need to be examined. Scientist are now using induced pluripotent stem cells (iPSC) derived from somatic cells to create transgenic animals. By using iPSC from goats for nuclear transfer, scientists were able to increase the survival rate of embryos and live births compared with ESC generated embryos (57). With increase efficiency of gene editing technologies, more research is being done to increase efficiencies of delivery methods.

Sperm-mediated gene transfer (SMGT) is also commonly used, and can transfer genes by binding exogenous DNA to the sperm which then transfer the genetic material to the egg during fertilization. This was first seen in rabbits in 1971 where it was shown that sperm cells could transfer foreign DNA that was bound to the head of sperm (44). Using sperm to transfer DNA has been shown to work at varying efficiencies in multiple species, in 1989 Lavitrano et al. had a success rate of 30% in mice but Sperandio et al. only saw 5.7% in swine (63-65). This method has been used in a variety of species including mouse (64,66-68), cow (2,65,69), and other species. The common method for SMGT is to incubate sperm with DNA and the sperm is either used *in vitro* fertilization system (64,65,68) or in artificial insemination (1,65,69).

1.10 Liposomes

Cationic lipids have been used to transfect DNA into cells since 1987 (70). Due to the fact that DNA has a negative charge, liposomes are used to surround DNA in order to create a positive charge. Felgner used N-1-(2,3- dioleyloxy)propylJ-N,N,N-trimethylammonium chloride (DOTMA) a cationic lipid that successfully introduced DNA into cells. Cells are commonly

transfected using anionic or cationic liposomes, cationic liposomes are used more often because anionic liposomes are time-consuming and complicated to construct. Cationic lipids have also been shown to have higher transfection efficiency. Studies have shown that when cationic lipids are complexed with DNA entrapment efficiency was ~100%, while non-cationic liposomes showed only ~50% (71,72). Liposomes have an advantage over other transfection methods because the only limitation on size is the ability of the cell to transcribe the introduced DNA. Cells have been transfected with bacterial YAC sequences that were 85 kilobase (kb) Yeast Artificial Chromosome (YAC) which contained a portion of a human heavy chain immunoglobulin gene and 650 kb YAC that encoded for human β-amyloid precursor protein (71,72).

Studies have been done to better understand how liposomes function in order to increase the efficiency DNA transfer. There are different theories on how liposomes deliver DNA into the cell and these are still being discussed. One hypothesis by Felgner et al. is that plasmid DNA is trapped inside the liposome (70). The liposome-DNA complex then fuses with the plasma membrane of the cell to deliver DNA into the cytoplasm. This theory was formed due to the observation that DNA complexed with cationic liposomes will fuse with negatively charged liposomes and the membranes of treated cells (70). This theory however, was disproved when it was shown that the fusion activity of cationic liposomes can be removed by pre-incubation of the cell with DNA. The study showed that the presence or absence of DNA did not affect the fusion of cationic liposomes (73). These varying results lead to questioning of the fusion theory. Further studies saw that that when liposome-DNA complex was incubated with the cells that the liposomes absorbed the cell surface resulting in destabilization of the plasma membrane, allowing translocation across the membrane (74). This method is still not fully understood.

The majority of liposomes contain a linear alkyl chain with polar and non-polar region. The bridge structure between these two groups may affect the cytotoxicity level (75). Most naturally occurring lipids are ester linked, Leventis and Silvius showed that ester link bonds facilitated greater degradation which allowed for greater transfection and less cytotoxicity (73). Ghosh reported different results than Silvius, and saw higher transfection efficiency when using an ether backbone compared to ester and urethane groups (76). Today, ether linked lipids are commonly used in commercial products such as Lipofectamine® and Lipofectin® (76) because the add stability and shelf life to these products.

One study was performed using the cationic lipid, 3-(trimethyl ammonium iodide) 1,2 dimystryl-propanediate (TAID) which contained an ester linked bond between polar groups in combination with a neutral lipid, DOPE (3). TAID was synthesized in a two-step process and resulted in a high product yield. They saw stable transfection in five different cell lines. The highest rate of gene transfer was seen when TAID:DOPE was used in a 2:1 molar ratio. This ratio was then used in combination with SMGT using GFP plasmid. They saw that 94% of day 8 embryos expressed the GFP gene, although they did not confirm if the integration of the transgene had occurred (3).

1.11 Gene Editing

In the past decade, a new approach of 'genome editing' has emerged in science. This technology uses engineered nucleases consisting of sequence-specific DNA-binding domains fused to a nonspecific DNA cleavage module. With this technology the nucleases can directly cut the DNA which then stimulates the endogenous DNA repair mechanism leading to non-

homologous end joining (NHEJ) or homology directed repair (HDR) (49). Due to this advancement in genetic engineering, producing transgenic animals has become more accessible.

Zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regulatory interspaced short palindromic repeats (CRISPRs) are the leading methods in genome engineering. ZFNs and TALENs are chimeric nucleases composed of programmable, sequence-specific DNA-binding modules linked to a nonspecific DNA cleavage domain as stated before (49). They can be customized to recognize almost any sequence but require complex engineering. CRISPRs are found naturally in bacteria and provide immunity against invading foreign DNA by using RNA-guided DNA cleavage. The technology uses the CRISPR-associated endonuclease (Cas9) to create a cut directed by guide RNA composed of 20 nucleotides which defines where the Cas9 will modify the genome (77). With the advent of ZFNs, TALENs and CRISPRs, there are more available tools to make introducing very specific modification into the genome possible, and there are likely to be many more genome engineering nucleases whose engineering potential we have yet to harness.

Zinc-finger nucleases (ZFNs) were the first gene editing technology that used programmable endonucleases. The Cys2-His2 zinc-finger domain is a binding motif that is found in eukaryotes and allows DNA binding. There are 30 amino acid domains in ZFN that can bind to DNA; usually 3 base pairs are designed to bind to a desired segment. ZFN target sites contain two ZFN binding sites, these are recognized by *Fok*I cleavage domain which cuts the DNA and results in HDR or NHEJ (78). These were first discovered in 1995, (79), but it was not until 2011 that they were used in livestock animals, rabbits (61) and pigs (80). In one study, rabbits were designed to have a disrupted endogenous IgM locus which is involved in the production of human polyclonal antibodies in the rabbit (61). In the pig, they knocked out α-1,3-

galactosyltransferase (GGTA1) gene as proof of concept that ZFNs can be used to create KOs in pigs for biomedical and agriculture use (80). ZFNs were used with homology directed repair in bovine fetal fibroblasts in order to generate a cow that secretes lysostaphin in milk to kill *Staphylococcus aureus*, a bacteria which causes mastitis (81). This was the first time ZFNs were successfully used to create a knock-in in a large animal species. While ZFN have proven to be effective tools in producing transgenic animals, constructing them can be difficult and timely.

TALENs were discovered in 2009 and proved to be easier to construct and effective in producing genetically engineered animals. Not only are TALENs easier to construct but they also have a greater specificity for binding to DNA. They consist of tandem repeated 34 amino acids, these contain residues 12 and 13, which were termed repeat variable diresidues (RVDs). This allows TALENs to bind to a single base pair unlike ZFN which are only capable of binding to three base pairs (82). TALENs were first reported to create KO in large animals in a study done in 2012 where they studied pigs and cattle. When the TALENs were used in pairs and directly injected as mRNA into the cytoplasm, indels were induced in both swine (30%) and bovine (75%) (83). Gene knock-ins were also created using TALENs. In one case, cattle were genetically engineer to be resistant to tuberculosis (84), and TALENs combined with homology directed repair were used to introduce the POLLED allele into horned bovine (85). One of the difficulties with gene editing is the risk of creating off-target effects that lead to unintended mutations. Nickases increase the specificity of the nuclease by requiring two targets in close proximity in order to make a cut. By using TALE nickases directed against the β-lactoglobulin (BLG) locus combined with human serum albumin (86) gene targeting vector, they were able to create homozygous cows that had higher levels of HSA in a more specific and safe manner (87). These gene editing technologies are becoming more efficient and are being used for designing

disease resistant livestock, improving animal welfare, and improving production value. Editing technologies continue to improve with the arrival of CRISPRs.

Clustered regulatory interspaced short palindromic repeats (CRISPRs) are the most recently discovered genome engineering technology (88). This system was discovered in a microbial adaptive immune system and has allowed great advancements because it is easy to design, does not take long to construct, is low cost and effective. The CRSIPR/Cas9 works by using Cas9 nuclease derived from Streptococcus pyogenes to cut the gene and a sgRNA to direct the nuclease. The sgRNA consists of a 20 base pairs (bp) that complement the target sequence that is adjacent to a protospacer adjacent motif (PAM) sequence. This simply means the sgRNA is next to a "NGG" trinucleotide motif (77).

Since the CRISPR/Cas9 technology is so new, it was initially largely used in mice and only recently has there been success in large animals. In 2014, the first report of using CRISPR/Cas9 system in large animals was achieved in pigs. The *vWF* gene was targeted to make a medical model that mirrored human deficiency that leads to von Willebrand disease. Cas9 mRNA and sgRNA were injected into zygotes and 63% of the embryos that survived contained the deletion (89). The next step was creating a knock-in by using a single strand oligo nucleotide (ssODN) combined with a homology directed repair (HDR) template. In pigs, zygotes were injected with Cas9 mRNA, sgRNA and ssODN to create founder pigs containing the desired HDR mutation with an efficiency as high as 80%; 7 pigs were born and 5 had the mutation (90). The CRISPR/Cas9 system has also been used in dairy cattle to insert the gene for human fibroblast growth factor 2 (FGF2) into exon 3 of the β-casein gene in order to produce FGF2 at a higher level so it can be used as a therapeutic agent (91). With the ease of this new gene editing technology the ability to create transgenic animals is now much more accessible, and the

applications are unlimited. Genome editing technology is only in early stages, and there are predicted to be countless editing agents whose potential we have yet to harness (92).

The goal of this experiment was to show that the CRISPR/Cas9 system could be used to make a single nucleotide change in the cattle genome. The α-LA gene sequence was chosen because a single nucleotide change has been identified to be linked to increase milk production in dairy breeds. A single nucleotide change in the cattle genome can be used to increase production of dairy cattle that live in regions where high producing dairy cattle, such as Holsteins, do not produce as well.

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Chapter 2

Creating a single nucleotide change in MAC-T and fetal fibroblast cells using CRISPR/Cas9 as a preliminary step to increase milk production in vivo

Abstract

In 2050, the expected size of the human population is ~9 billion, the demand for food will increase, and the demand for milk will increase along with it. Genetically modifying animals is a tool that can be used to meet this growing demand. In the United States, Holstein cows are the leading breed for milk production. They produce on average ~24,291 pounds of milk per year, whereas Jerseys, the other major dairy breed, produce on average ~16,997 pounds. Holstein cows ability to produce large quantities of milk is attributed to multiple factors, one of them being selection for superior genetics. An example of this can be seen in the α-lactalbumin (α-LA) gene sequence. The α-LA gene sequence in Holstein cows contains a mutation at the (+15) position which corresponds to the transcriptional start point of α-LA (+1). Holstein cows with this mutation have an adenine at this position, in contrast to Holsteins without this mutation and other cattle breeds with lower milk production, which have either a cytosine or guanine. The purpose of this experiment was to determine if the CRISPR/Cas 9 system could create a single nucleotide change in a bovine cell line. CRISPRs were used because the CRISPR/Cas9 system is inexpensive, easily programmed, and efficient. In this study, we worked with Holstein MAC-T cells and Angus fetal fibroblasts. Cells were then transfected with the designed CRISPRs by a variety of transfection methods, including FuGENE® 6 (Promega, Madison, WI, USA), electroporation, and Lipofectamine® 2000 (ThermoFisher Scientific, Waltham, MA, USA). Cells were transfected with a Cas9 plasmid, pSpCas9(BB)-2A-GFP, and a ssODN to insert the desired mutation. The plasmid DNA contained a GFP selection marker, cells were transfected and sorted using FACS. MAC-T cell DNA was analyzed using enzyme digestion with BpuEI, while the

Angus fetal fibroblasts were analyzed using the surveyor assay. The CRISPR/Cas9 system was able to create a double strand break in the desired region of the MAC-T cells resulting in indels and deletions, but we were not successful in creating a single nucleotide change. However, we did see a single nucleotide change in Angus fetal fibroblasts using CRISPRs and ssODN.

2.1 Introduction

In the past 40 years, milk production in dairy cattle has more than doubled. The majority of this increase has been due to genetic selection (1). Although this increase is substantial, the demand for milk will only increase as the population continues to expand. Genetically modifying animals is a tool that can be used to meet this growing demand. In the United States, Holstein cows are the leading breed for milk production. To better understand why Holsteins had such an advantage in producing milk, Bleck and Bremel sequenced a variety of beef and dairy cattle genomes and looked at different gene sequences involved in milk production (2). They found a single nucleotide change in the 5' promoter region of the α-lactalbumin (α-LA) gene sequence. This mutation was at position (+15) from α-LA exon (+1), which corresponds to the transcription start point of α-LA. Holstein cows have an adenine at this positions in contrast to the other cattle breeds with lower milk production, which have a guanine. The α-LA sequence was specifically targeted as a gene of interest because α-LA plays a key role in milk production (3). α-LA combines with galactosyltransferase to synthesize lactose in the Golgi apparatus (4). Lactose is a major sugar in milk, once it is synthesized it functions as an osmotic regulator in milk secretion (5). By increasing production of α-LA, the amount of lactose production increases which leads to greater production of milk. Cows with α-LA (+15) AA (an adenine at both alleles) had higher milk production compared to α-LA (+15) BB ($P < 0.01$) (a cytosine, guanine,

or thymine on both alleles), while animal heterozygous at the locus, α-LA (+15) AB had a value in-between that of α-LA (+15) AA and α-LA (+15) BB (6).

The single nucleotide change from a guanine to an adenine occurs in the 5' flanking sequence of the promoter region. Although it has not been proven, it is believed that this region is associated with the Nuclear Factor 1 (NF1) family. NF1 was purified from HeLa cell nuclear extracts and were shown to be involved *in vitro* replication of adenovirus DNA, further studies showed they recruit viral replication proteins to the replication origin and that it targets the DNA binding domain of the protein (7-9). This may be involved in mammary development since many of the genes are activated at later stages. When they looked at the 5' flanking region of α-LA in rats, they found two binding sites that were highly conserved. One sequence was TTGGCAG, which is very similar to the NF1 binding region TGG(C/A)N_5GCCAA (10) (11-13). The adenine in Holsteins may increase the binding affinity for the NF1 family. By combining this knowledge with gene editing technologies we have the ability to improve production in livestock animals.

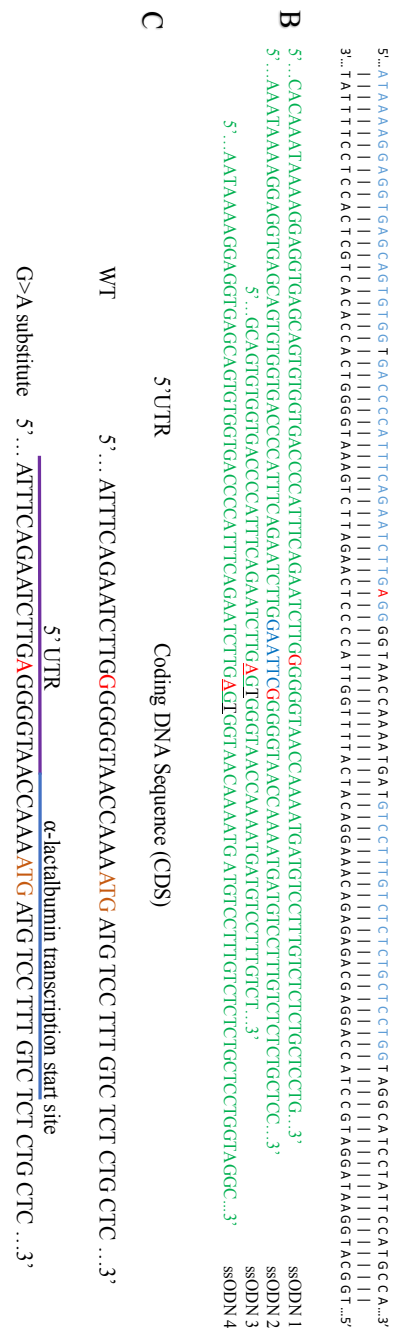
Clustered regulatory interspaced short palindromic repeats (CRISPRs) are a new gene editing technology that allow more specific gene targeting. The CRISPR/Cas9 system was discovered in bacteria which use it as a defense mechanism against invading pathogens (14). It functions by using a guide RNA (sgRNA) consisting of 20 bp, to guide the Cas9 nuclease to the desired location on the DNA and create a double strand break. For the CRISPR to target the desired sequence the sgRNA need to be adjacent to a protospacer adjacent motif (PAM) sequence, which refers to "NGG" trinucleotide motif (14).This cut is then repaired by the error-prone non-homologous end joining (NHEJ), or by homology directed repair (HDR). By combining the CRISPR/Cas9 system with a repair template the desired mutation can be inserted by HDR. This can be done using a single strand oligo nucleotide (ssODN) as a template (14).

For this experiment, three sgRNA were designed to create a cut near the (+15) position of the α-LA gene sequence (Figure 1C). In order to determine the cutting efficiency of the CRISPR, mammary epithelial cells (MAC-Ts) and Angus fetal fibroblasts were used. MAC-T cells are an immortal cell line derived from bovine mammary epithelial cells. This cell line was made immortal using SV-40 large T-antigen to circumvent senescence. It maintains the morphological characteristics of mammary epithelial cells as well as the ability to produce lactogenic hormones and form an extracellular matrix, which is important for studying milk synthesis (15). The Angus fetal fibroblasts contain the wild-type nucleotide, guanine, at the (+15) position form the α-LA transcriptional start point. Successful gene editing in fetal fibroblasts provides a suitable edited cell line for later use of nuclear transfer.

CRISPR plasmids were designed to create a double strand break in the cell lines by cloning three 20 nucleotide target sites into the gRNA scaffold. These plasmids containing the Cas9 protein also contained GFP or the puromycin selection markers. Cells were then transfected using FuGENE® 6, Lipofectamine® 2000, or electroporation. While Lipofectamine® 2000 and electroporation had a higher transfection efficiency, FuGENE® 6 had a greater survival rate. Similarly, selecting cells using GFP combined with flow cytometry was the least toxic method for selecting cells, compared to puromycin. sgRNA2 was closest to the mutation and cut with a 15% efficiency in MAC-T cells and a 50% efficiency in Angus fetal fibroblasts. The desired single nucleotide change was not seen in MAC-T cells, but a single colony of fetal fibroblasts out of 14 colonies did have contain the desired mutation due to the insertion of the designed ssODN.

Figure 1: Introduction of G>A substitution of 5' flanking region of α-LA promoter.

(A) A schematic illustration showing the location of the gRNAs. Blue bars and letters indicate the position of the gRNA targets with the orange bars label the PAM sequence. The mutation that is associated with increased milk production is in red letters. (B) ssODN 1 and 2 contain 45 bp homology arms flanking either side with a guanine at the (+15) position. ssODN 2 has a ECORI site (GAATTC). The second two have an adenine at the (+15) position. ssODN 3 has 30bp homology arms flanking the mutation, ssODN 4 has 45 bp homology arms. (C) Representation of the α-LA gene sequence wild type and with a single nucleotide change.



2.2 Materials and Methods

Generation of CRISPR Plasmid

The gRNA/Cas9 dual expression vector pSpCas9(BB)-2A-Puro (PX459) (Figure 2A) and pSpCas9(BB)-2A-GFP (PX458) (Figure 2B) were obtained from Addgene (Cambridge, MA). Three sets of 20 nt guide RNA were ordered from IDT (Coralville, Iowa) (Figure 1A) and inserted into PX459 and PX458 plasmid using molecular cloning with restriction enzymes and T4 ligase using the CRISPR/Cas9 system protocol (16). Plasmid DNA was transformed into Stellar Competent cells (E. Coli) from Takara (Mountain View, CA) and cultured overnight. Plasmids were isolated using GeneJET Plasmid miniprep kit (Thermo Fisher Scientific, Waltham, MA) and sent to sequencing at UIUC Core Sequencing Facility (Urbana, IL) with U6 forward primer to detect if the sgRNA had successfully inserted into the plasmids.

Figure 2: Plasmids

(A) pSpCas9(BB)-2A-Puro used for transfection, contained puromycin for antibiotic selection.

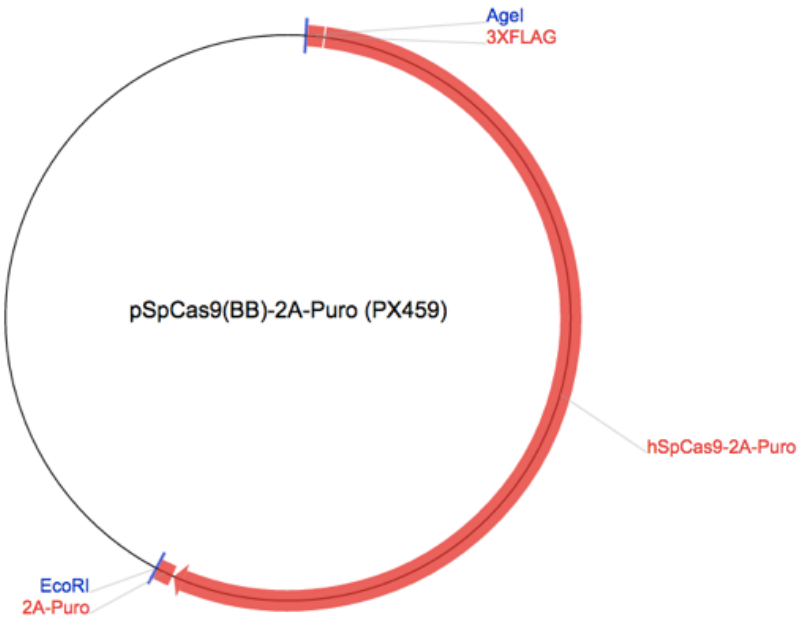
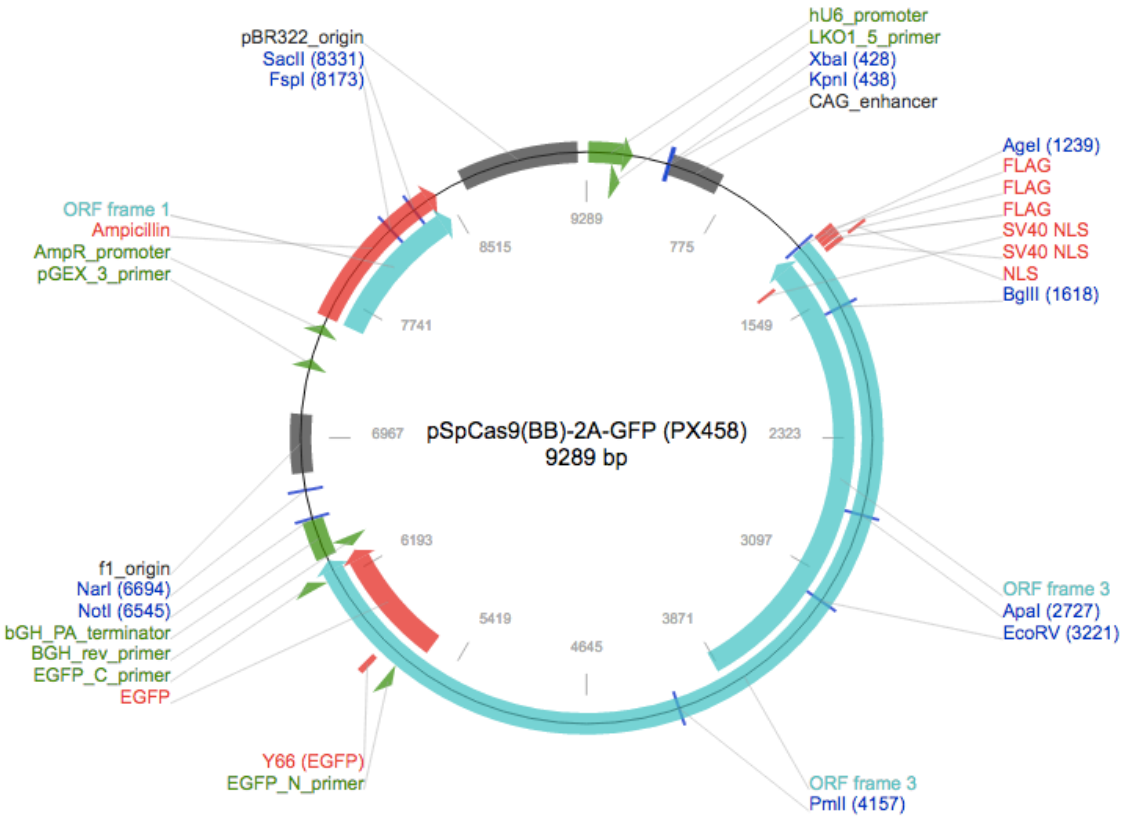


Figure 2: Plasmids (cont.)

(B) pSpCas9(BB)-2A-GFP used for transfection, contained GFP. Both plasmids were from the Zhang Lab and purchased from Addgene (Cambridge, MA).



ssODN Design

Single strand oligo nucleotides were designed and ordered from IDT (Coralville, Iowa). The first two templates designed contained a guanine at the (+15) position and contained 45 base pairs on either side of the mutation, and one also included and ECOR1 (GAATTC) cut site immediately upstream of the guanine at (+15) position. The next two ssODN that were designed contained an adenine at the (+15) position with a mutation in the PAM sequence, meaning NGG was changed to NGT. This was to prevent the re-cutting of the DNA once the sequence had been

inserted. One was designed with 30 base pair homology arms and the other was designed with 45 base pair homology arms (Figure 1B).

MAC-T Cell Transfection

MAC-T cells (3) were cultivated in 75 cm^2 flasks in DMEM with 10% fetal bovine serum (FBS), 1 ml/L Pen/Strep, 50mg/L gentamycin and 1 mL/L Amphotericin B. Media was changed every other day. 150,000 cells were plated the day before transfections in a 6-well plate with DMEM (high glucose, Sigma-Aldrich, St. Louis, MO), 10% FBS and no antibiotics. Once they reached 70% confluency, MAC-Ts were transfected with FuGENE® 6 Transfection Reagent (Promega, Madison, WI). Two μg of DNA were added to 100 μl of opti-MEM (Thermo Fisher Scientific, Waltham, MA), then 6 μl of FuGENE® 6 Transfection Reagent were added to the Opti-MEM and left at room temperature for 15 minutes. When MAC-T cells were transfected with ssODN, the first sort with ssODN 1 and 2 contained 1 μg of CRISPR plasmid DNA and 1 μg of ssODN. The second sort using ssODN 3 and 4 used 10 or 30 picomoles (pmol) and CRISPR plasmid DNA to have a total of 2 μg of DNA. The mixture was then added dropwise to the well and put in the incubator. After 24 hours, the cells were imaged using a florescent microscope Olympus IX71 to determine the percent transfected.

Angus Fetal Fibroblast Transfection

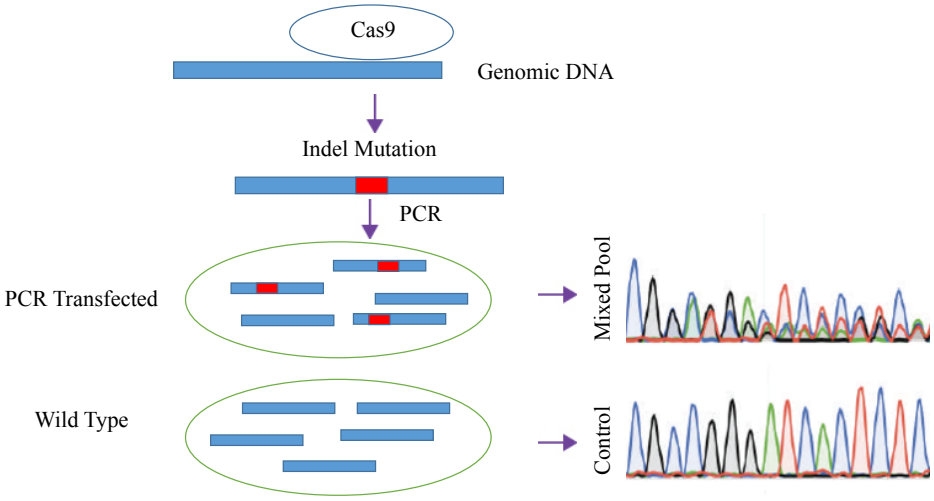
The Angus fetal fibroblasts used (a generous gift from Dr. Jonathan Beever, Urbana, IL) were grown in DMEM/F10 media (Cell Media Facility, Urbana, IL) supplemented with 10% FBS, 0.01μg/mL basic fibroblast growth factor (bFGF) (Sigma-Aldrich, St. Louis, MO), 1 ml/L Pen/Strep and 1 mL/L Amphotericin B. Transfection was carried out as stated previously using FuGENE® 6 Transfection Reagent.

Verification of sgRNA with pSpCas9(BB)-2A-Puro

Cells were transfected with pSpCas9(BB)-2A-Puro using FuGENE® 6. Twenty-four hours post transfection, puromycin selection was used to select cells that had taken up the Cas9 plasmid. This is a method of antibiotic selection, cells that have taken up the plasmid will be resistant to puromycin. Based on a kill curve the appropriate amount of puromycin was added to select transfected cells. One μg per milliliter of puromycin was applied for selection over two days. Media was changed after 48 hours, cells were then grown until they reached confluency, then genomic DNA was extracted using Wizard® Genomic DNA Purification Kit (Promega, Madison, WI). TIDE (Tracking of Indels by DEcomposition) was used to compare the transfected Mac-T cells to normal Mac-T cells. TIDE software measures the editing efficiency and looks for insertions or deletions (indel) mutations created by CRISPR in pools of gDNA by comparing wild type cells to transfected cells in order to quantify the editing efficacy of CRISPR/Cas9 system (Figure 3) (17).

Figure 3: CRISPR/Cas9 system schematic diagram.

Cas9 nuclease binds to the DNA represented as a blue bar and creates a cut. When the DNA repairs itself it results in an indel which can be an insertion or deletion. PCR products are sent to sequencing, and then analyzed using TIDE which compares sequences from a mixed DNA pool.



MAC-T Gene Edit Analysis

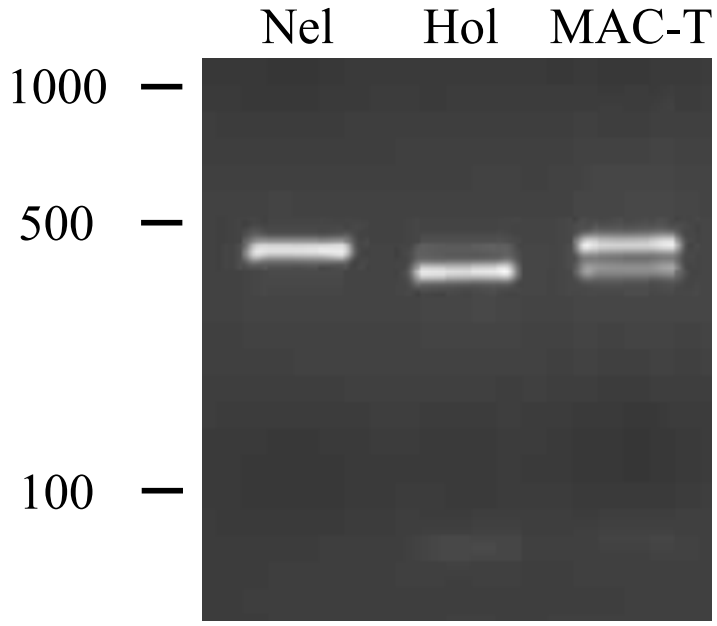
MAC-T cells were also selected by using flow cytometry. This method used pSpCas9(BB)-2A-GFP (PX458), which is a plasmid containing a green fluorescent protein (GFP) as a marker. If the cells took up the plasmid DNA they would fluoresce green, this fluorescence can be detected by a machine, which then sorts the cells into wells. After MAC-T cells were transfected with pSpCas9(BB)-2A-GFP (PX458) they were taken to the Flow Cytometry Facility (Urbana, IL) where Fluorescence Activated Cell Sorting (FACS) was used to sort 5 cells into a 96-well plate with DMEM (high glucose, Sigma-Aldrich, St. Louis, MO), 10% FBS, 1 ml/L Pen/Strep, gentamycin 50mg/L and 1 mL/L Amphotericin B. Once cells reached confluency they were trypsinized and moved into a 24-well plate. After reaching confluency, the cells were trypsinized, some were added back to the well and the remaining cells were

centrifuged. Fifty µl of QuickExtract™ DNA Extraction Solution (Epicentre, Chicago, IL) was added to the cells then placed at 65°C for two hours and finish with 98°C for 10 minutes.

The α-LA gene sequence was analyzed by conventional PCR using α-LA (+15) forward primer (TGGACCCTT TGTGCATTTTCT) and α-LA (+15) reverse primer (TGGGTGGCATGGAATAGGAT) resulting in 420 bp. These were combined with JumpStart™ Taq DNA Polymerase (Sigma-Aldrich, St. Louis, MO) and cycling parameters: 30 sec at 94°C, 45 sec at 60°C, 45 sec at 72 °C, second two 30 times, 10 min 72°C, and 5 min 4°C. The PCR product was digested with restriction enzyme BpuEI (CTTGAG) (New England BioLabs, Ipswich, MA) at 37°C for one hour. Then the cuts were then analyzed on a 2% agarose gel. MAC-T cells are heterozygous for the mutation at the (+15) site; they have one copy of the allele with adenine and one with guanine. Non-edited cells show three bands, one at 420 bp and one at 370 bp, and a very faint band at 50 bp, cells that were edited have a frameshift or deletion and result in one band at 420 (Figure 4). Cells that showed two bands were discarded, while cells that had one band were transferred to a 6-well plate and frozen -80 in freezing media 80% DMEM with 10% FBS, 10% Dimethy Sulfoxide (DMSO) (Thermo Fisher Scientific, Waltham, MA), and 10% FBS.

Figure 4: BpuEI digestion

DNA was extracted from Holstein blood (Hol), MAC-T cells (MAC-T), and Nelore cells (Nel). The DNA was amplified by PCR and then digested with BpuEI which creates a cut at CTTGAG. The DNA fragments were separated on a 2% agarose gel. Holstein cows have the adenine mutation and are cut showing a band at 370 bp. MAC-T cells are heterozygous for the mutation and have a band at 420 bp, 370 bp and a very faint band at 50bp. Nelore do not have the adenine mutation and no not get cut, showing a band only at 420 bp.



Angus Fetal Fibroblast Gene Edit Analysis

Fetal fibroblasts were sorted by the same method. They were grown in DMEM/F10 media until they reached confluency in a 24-well plate. DNA was extracted using QuickExtract™ DNA Extraction. α-LA (+15) expression was analyzed using Herculase II Fusion DNA Polymerase (Agilent Technologies, Santa Clara, CA), with the same α-LA(+15) primers used for MAC-T cells cycling parameters: 2 min at 95°C, 15 sec at 95°C, 20 sec at 52.5°C, 30 sec at 72°C, go to step 2 and repeat 31 times, and then 3 minutes at 72°C. The PCR product was then size separated on a 2% agarose gel and DNA was then extracted from the desired band at 420 bp using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Waltham, MA). Surveyor assay (IDT, Coralville, Iowa) was used to determine the targeted

cleavage efficiency. The surveyor nuclease is part of the CEL family and recognizes mismatches between non-edited and edited strands and created a cut at those positions. Non-edited cells show a single band at 420 bp, edited cells show multiple bands.

Electroporation

MAC-T cells were electroporated using Gene Pulser® II Electroporation System with an attached capacitance extender. The voltage capacitance was set at 950 μF, with four different voltages, 250V, 300V, 350V, and 400V. Five million or two million five hundred thousand MAC-T cells were suspended in 400 μl of Opti-MEM and placed in a tube with 10 μg of plasmid GFP DNA which was then transferred to a cuvette and loaded onto the Gene Pulser. Cells were then placed in a 6-well plate, after 24 hours media was changed and cells were imaged to determine the efficiency. This was repeated with five million cells at 100V, 150V, 200V, and 220V in 400 μl of Opti-MEM with 10 μg of DNA. Once voltage was optimized, we changed that amount of capacitance. Two million five hundred thousand MAC-T cells were put in tubes with 400 μl of Opti-MEM and 10 μg of DNA. The cells were pulsed at a voltage of 220V or 150V and a capacitance of 350 μF, 500 μF, or 950 μF. After the medium had been changed 0.5 μg/ml of puromycin was added to each well for selection. Selection was applied for two days, after selection, cells were given time to recover and reach confluency followed by DNA extraction.

Lipofectamine

Two-hundred thousand MAC-T cells were plated 24 hours before lipofection in a 6-well plate with DMEM, and 10% FBS. Lipofectamine® 2000 (Thermo Fisher Scientific, Waltham, MA) was added at 6, 7, 7.5,8, 9, and 10 μl into 100 μl of opti-MEM. At the same time 2.5 or 3

µg of DNA were also added to 100 µl Opti-MEM, these two amounts were combined for 20 minutes and then placed in the 6-well plate that contained cells that were 70% confluent in DMEM with no FBS and antibiotics. After 6 hours the media was removed and replaced with DMEM, 10% FBS, 1 ml/L Pen/Strep, gentamycin 50mg/L and 1 mL/L Amphotericin B. One replicate was done with GFP plasmid DNA to determine which amount was most efficient. Another replicate was done using CRISPR plasmid containing puromycin resistance which was used to select transfected cells. After the medium had been changed, 0.5 µg/ml of puromycin was added to each well for selection. This was done for two days; cells then grew back and DNA was extracted.

2.3 Results

MAC-T Cell Transfection

MAC-T cells were first transfected with FuGENE® 6 with pIRES2-EGFP, resulting in 30% fluorescence and 80% of the cells were still attached 24 hours after transfection. Lipofectamine® 2000 was then used in attempt to increase the transfection efficiency, this resulted in a wide range of fluorescence (20-40%), and a large amount of cell death, only 30% of cells were still attached 24 hours after selection. Lipofectamine® 2000 proved to be too toxic for MAC-T cells. Electroporation was also experimented with to increase transfection efficiency (30-40%), this method also resulted in massive cell death, with 40% of cells still being attached 24 hours following transfection. FuGENE® 6 was used for the following experiments.

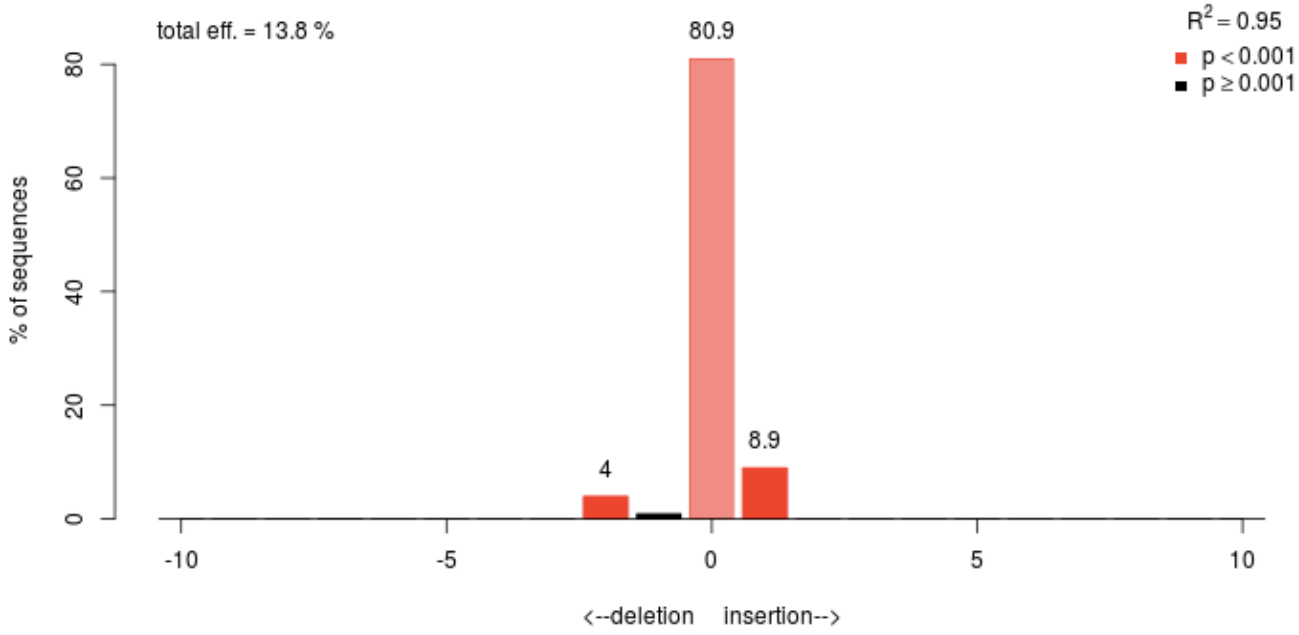
Validating sgRNA with pSpCas9(BB)-2A-Puro

The purpose of this experiment was to test the cutting efficiency of candidate clustered regularly interspaced short palindromic repeats (CRISPRs) that will later be used in knock-in

experiments. Cells transfected with pSpCas9(BB)-2A-Puro were selected using puromycin, once the cells had recovered, extracted DNA product was sent to sequencing and analyzed by tracking of indels by Decomposition (TIDE) created by the Bas van Steensel lab (Netherlands Cancer Institute). Only gRNA 2 was analyzed, TIDE results reported a 13.8% cutting efficiency in MAC-T cells (Figure 5).

Figure 5: Results from TIDE Analysis

The total efficiency as evaluated by TIDE was 13.8%.



sgRNA with pSpCas9(BB)-2A-GFP and ssODN

To generate a single edited cell line, pSpCas9(BB)-2A-GFP was used to transfect MAC-T cells using FuGENE® 6. The first approach was transfecting MAC-T cells with three different gRNA. Surveyor assay could not properly identify sequences that had been edited because MAC-T cells are heterozygous at the (+15) position. This led to the use of BpuEI for enzyme digestion which cuts when the mutation is present (Figure 6). This method could not be used to

determine if gRNA 1 and gRNA 3 work because the point at which they cut was too far from the position of the mutation and indels at these cut sites would not affect the efficiency of enzyme cleavage. In the first sort, sixteen wells in the 96-well plate survived and three of the colonies showed indels, one had a knock-out at the (+15) position (Figure 7). This confirmed that sgRNA can create a cut near the site of the mutation.

Figure 6: BpuEI enzyme digestion on sorted cells

MAC-T cells were transfected with pSpCas9(BB)-2A-GFP, fluorescent cells were sorted into a 96-well plate. DNA was extracted from cells, the DNA was amplified by PCR and digested by BpuEI. From this gel, DNA in well 4 did not contain the mutation and was sent to sequencing. DNA in well 9 is a negative control from Nelore cells and does not have the adenine mutation.

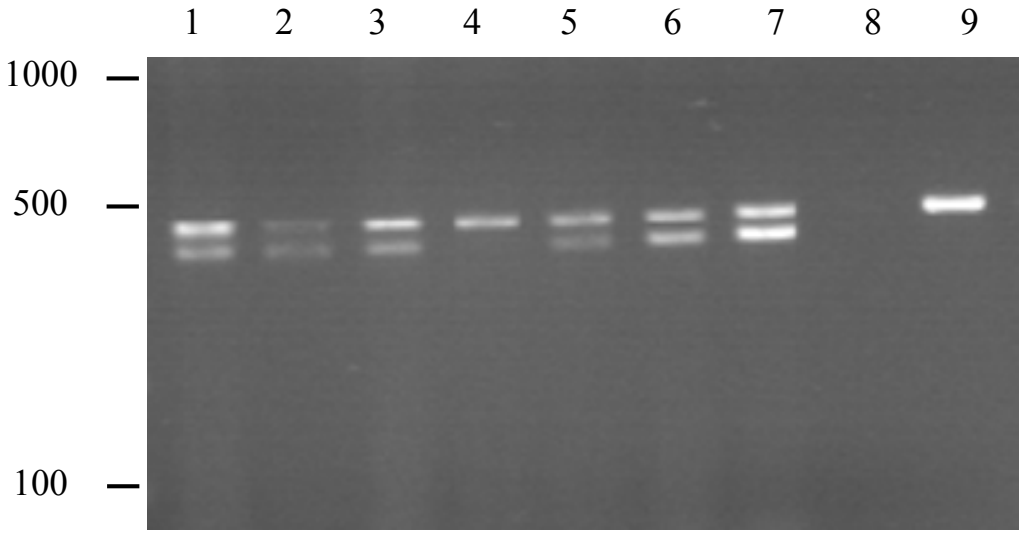
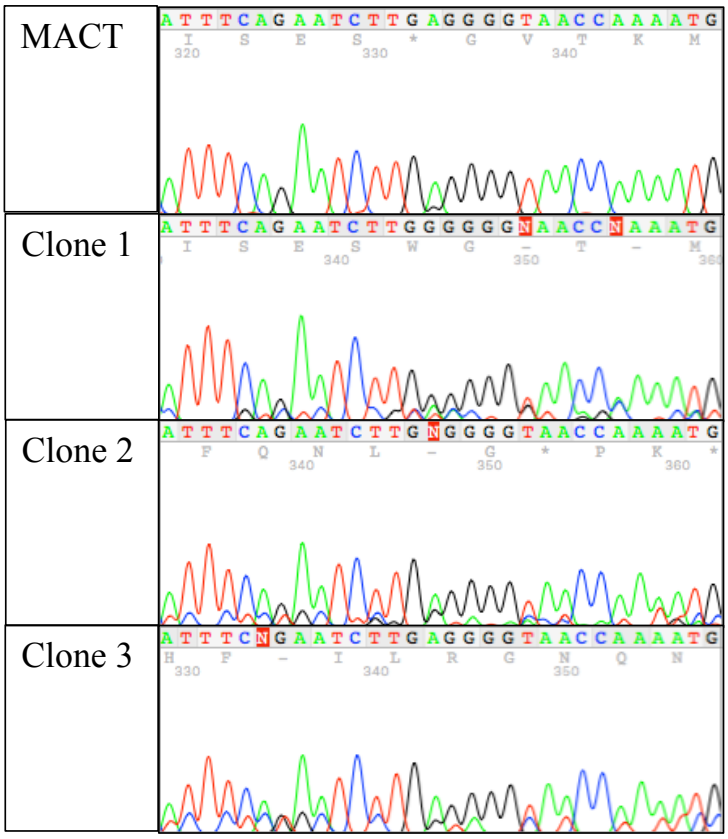


Figure 7: Chromatographs from first sort edited MAC-T cells with gRNA 2

The first chromatograph is from wild type MAC-T cells, the following three are from clones that show indels and frame shifts near the desired location. Clone 2 and 3 both have a knock-out of the adenine mutation.



With validation that sgRNA 2 can guide the Cas9 nuclease to the desired position, ssODN 1 and ssODN 2 were used in combination with the CRSIPR/Cas9 system. ssODN 1 had a guanine in the (+15) position with homology arms that were 45 base pairs in length. ssODN 2 was the same as ssODN 1 with the addition of EcoRI (GAATTC) sequence inserted in front of the guanine at the (+15) position. One µg of ssODN was added with one µg of pSpCas9(BB)-2A-GFP. For the gRNA 2 with no ssODN, five of thirty-three colonies that survived had indels (14.3% cutting efficiency) (Figure 8). The CRISPR combined with ssODN 1 had four out of thirty-five cells that showed indels (11.4% cutting efficiency) but none of them had a single nucleotide change. The final combination of ssODN 2 with CRISPR had 36 MAC-T cells that

survived and one of them showed an indel (3.8% cutting efficiency), but not the desired single nucleotide change (Figure 9) (Table 1).

Figure 8: Chromatographs from the second sort edited MAC-T cells with gRNA 2. Chromatographs show large frameshifts.

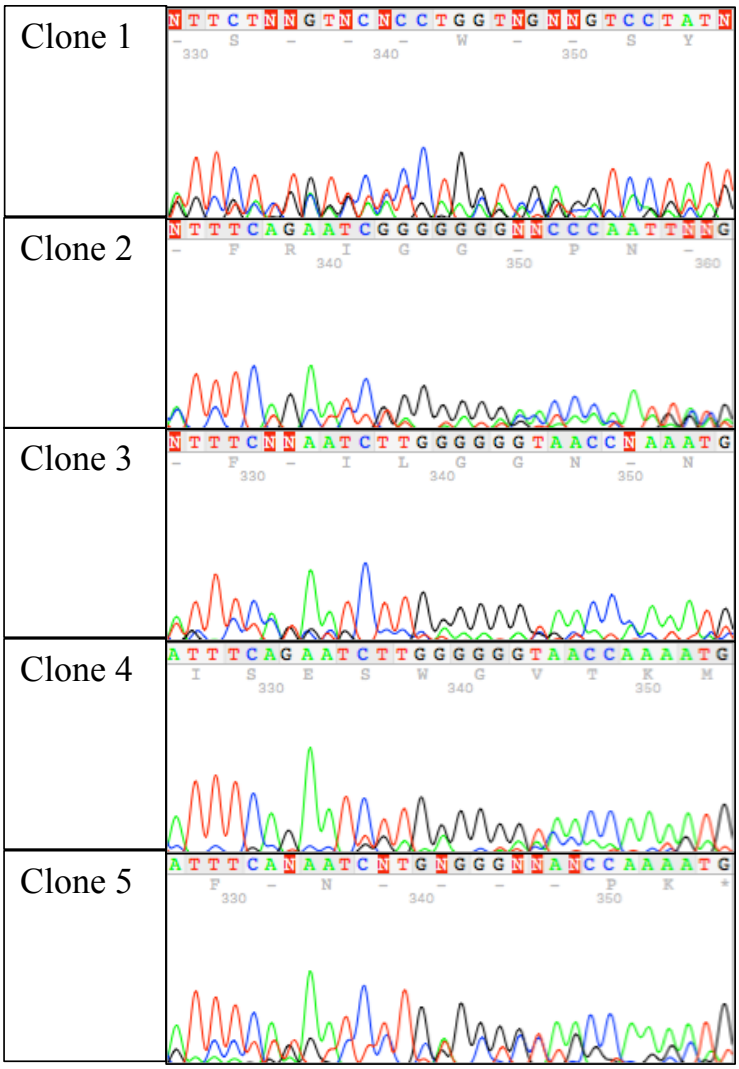
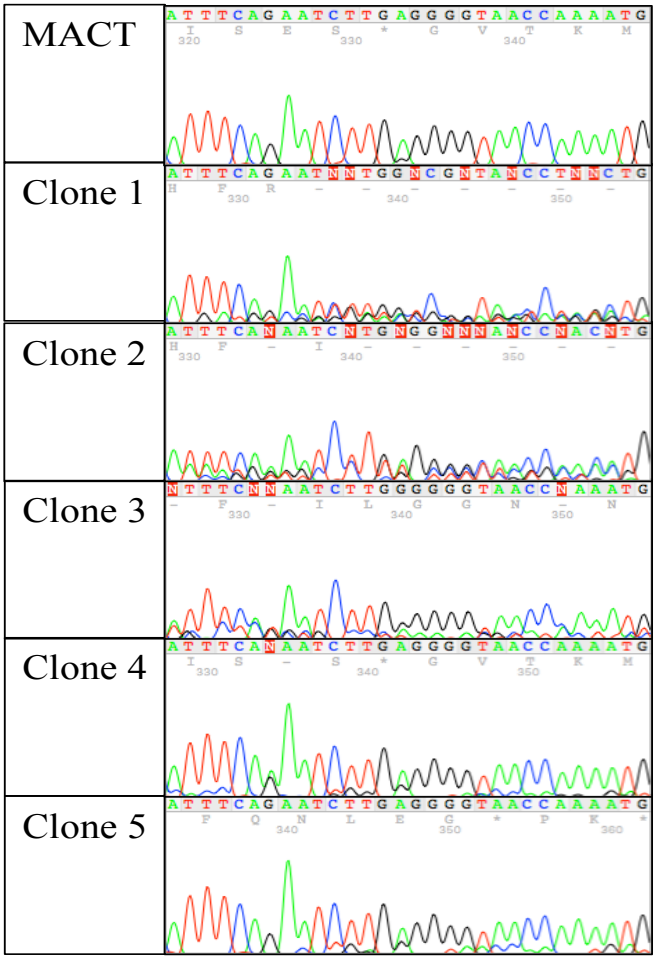


Figure 9: Chromatographs from the second sort edited MAC-T cells with gRNA 2 and ssODN

Chromatographs show large frameshifts.



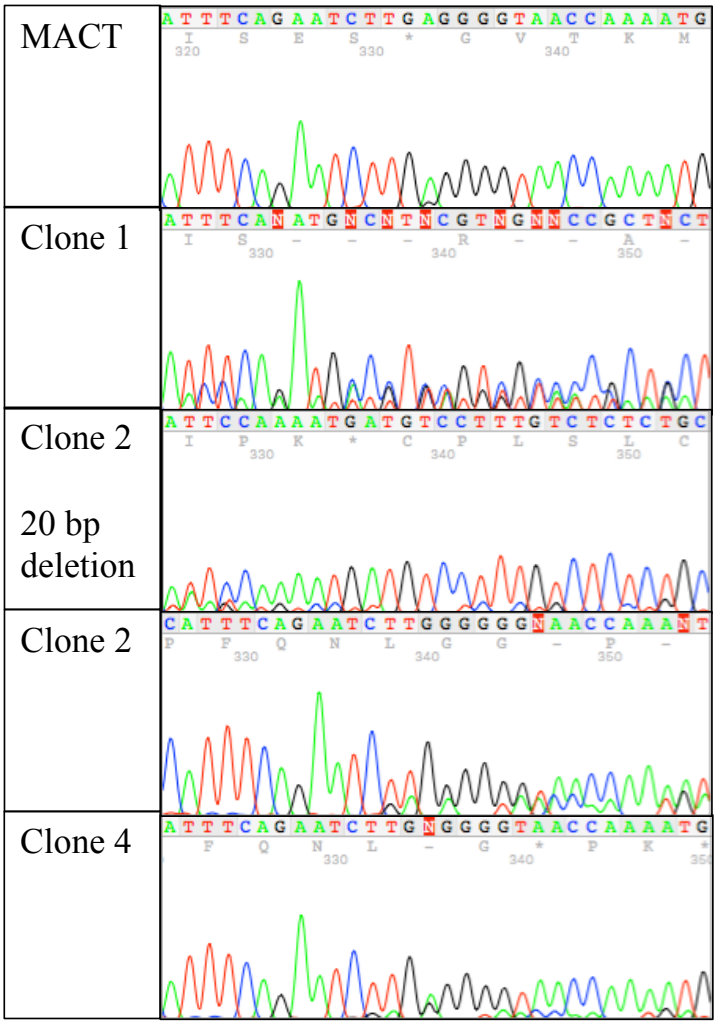
New ssODN were designed, containing an adenine at the (+15) position and a mutation in the PAM sequence. ssODN 3 was designed with 45 base pairs flanking the mutation and ssODN 4 had homologous arms that were 30 base pairs in length. The first experiment added 10 pmol of ssODN 3 and 4 with pSpCas9(BB)-2A-GFP with a total of two micro grams of DNA in two separate wells. And 30 pmol of ssODN 4 with pSpCas9(BB)-2A-GFP with a total of two micrograms of DNA in two separate wells. These were sorted into 96-well plates; 10 pmol of ssODN 3 was 38.7% fluorescent but only one clone survived. 10 pmol of ssODN 4 was 34%

fluorescent and had 7 clones survive. 30 pm of ssODN 4 resulted in 19.6% fluorescence and 37 clones survived (Table 1).

This experiment was repeated with 30 pmol of ssODN 4 which had the highest survival rate previously. Three wells in a 6-well plate were transfected then sorted into a 96-well plate. The wells were 15.6%, 12.9%, 5.1% florescent with a total of 42 clones formed. Ten colonies of clones died before DNA could be extracted, of the remaining cells that survived four showed edits using enzyme digestion (12.12%) (Table 1), these edits were confirmed through sequencing (Figure 10).

Figure 10: Chromatographs from the third sort edited MAC-T cells with gRNA 2 and ssODN

Clone 2 contains a 20 bp nucleotide deletion. Other clones show frame shifts.



Angus fetal fibroblasts were also transfected with pSpCas9(BB)-2A-GFP sgRNA 2and ssODN. In the first experiment, fetal fibroblasts were transfected with pSpCas9(BB)-2A-GFP and then sorted. The cells were evaluated using surveyor assay (Figure 11). Six clones survived and three of them had indels (50% cutting efficiency) (Figure 12). In the next experiment, fetal fibroblasts were transfected with 30 pm of ssODN 4 and pSpCas9(BB)-2A-GFP to reach a final volume of two micrograms. Two wells in a 6-well plate were transfected; 24 hours after

transfection the cells were pooled and taken to the flow sorting facility. The pooled cells were 8.2% fluorescent, these cells were then sorted into two 96-well plates with 5 cells per well. A total of 31 clones attached to the plate. Of those 31 colonies, only 7 became confluent and were transferred to 24-well plates (Table 1). DNA was extracted from colonies, DNA was then amplified using PCR, the PCR product was digested with BpuEI, DNA from one colony was successfully cut, which signifies the presence of the adenine mutation (Figure 13). This colony was sent to sequencing, which confirmed that the ssODN had inserted into the cell line (Figure 14). Surveyor assay was performed on the remaining DNA, of which none were edited.

Figure 11: Surveyor Assay

Angus fetal fibroblasts were transfected with pSpCas9(BB)-2A-GFP and sorted into a 96-well plate. DNA was extracted and then The DNA was amplified by PCR, the samples were evaluated using Surveyor Assay. Samples 2,4, and 5 have multiple bands showing they may have edits. The samples were sent to sequencing.

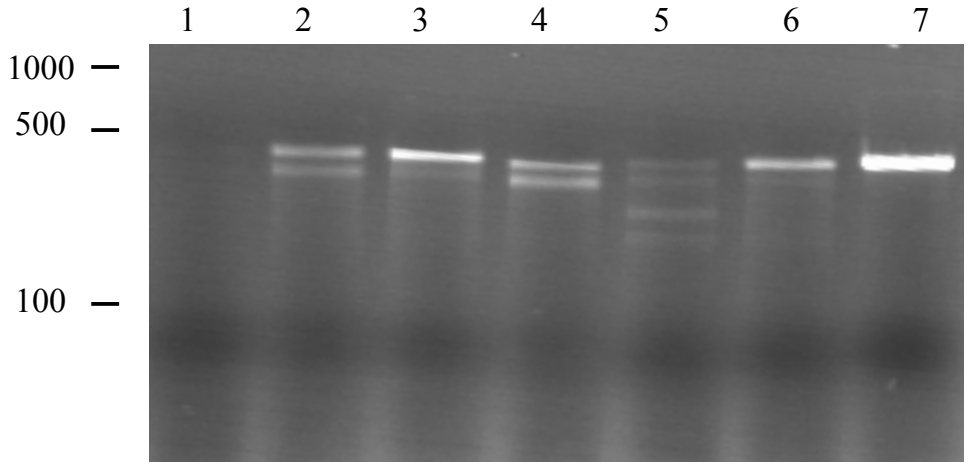


Figure 12: Chromatographs from second sort edited Angus Fetal Fibroblast cells with gRNA 2

Chromatographs show large frameshifts.

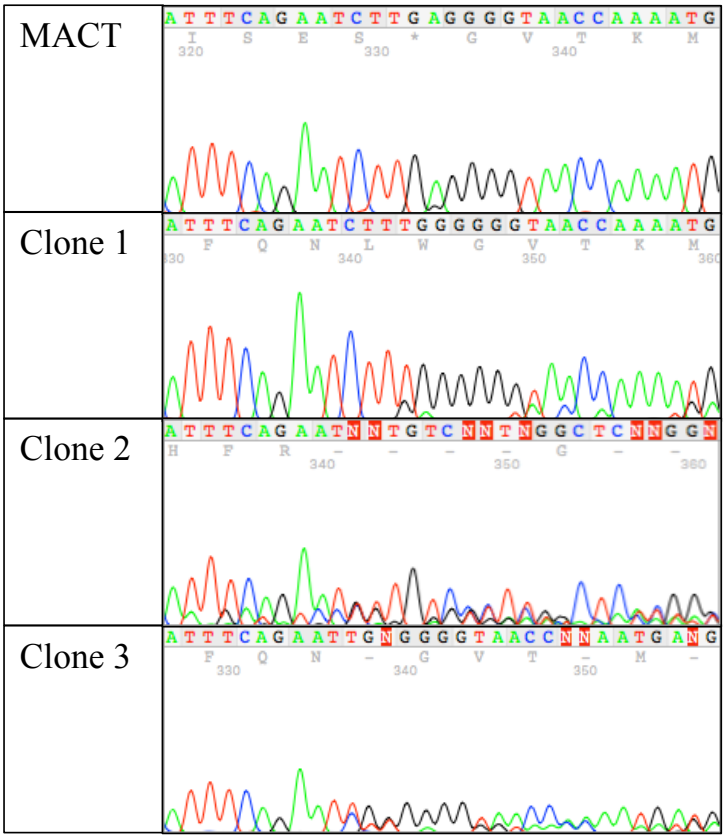


Figure 13: BpuEI enzyme digestion on Angus Fetal Fibroblasts

Fetal fibroblasts rom second sort were digested with BpuEI to determine if HDR had occurred using the ssODN. Colony 6 appears to contain the mutation. DNA was sent to sequencing.

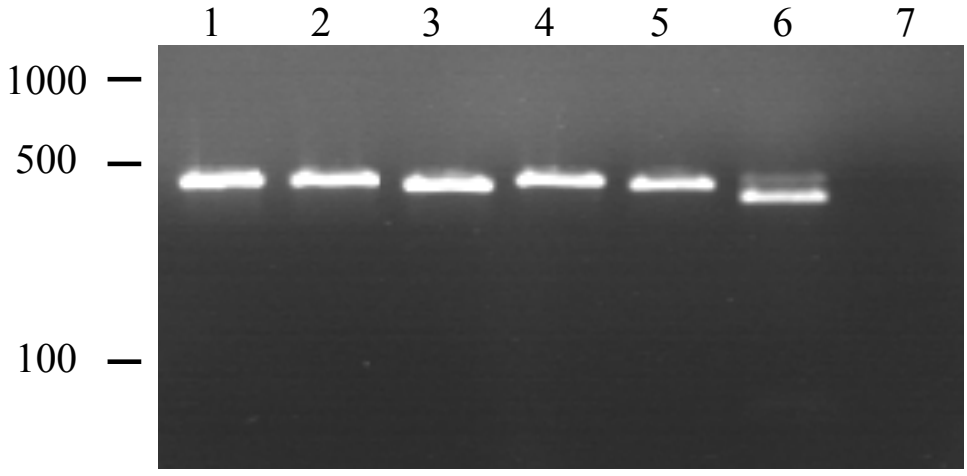


Figure 14: Chromatographs from second sort edited Angus Fetal Fibroblast cells with gRNA 2

This fetal fibroblast sequence changed from CTTGGGGGT to CTTGAGTGT. The adenine is the desired mutation and the thymidine in the PAM sequence shows that the single nucleotide change occurred because of the ssODN.

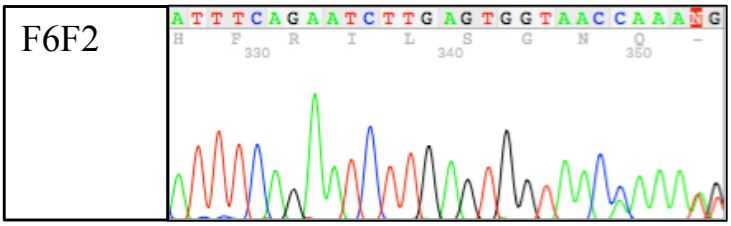


Table 1: Cell sorting

This table gives a list of the five sorts that were done with MAC-T cells or Angus fetal fibroblasts. Cells were transfected with pSpCas9(BB)-2A-GFP, 24 hours after transfection they were sorted using FACS. The %GFP was measured by the machine.

Sort	Cell Type	Method	% GFP	% Survival	%Indels
1	MAC-T	gRNA2	19.8%	16.67%	18.75%
2	MAC-T	gRNA2	12.7%	23.96%	21.74%
	MAC-T	ssODN	7.1%	27.08%	15.38%
	MAC-T	EcoRI	7%	30.21%	3.45%
3	MAC-T	ssODN	38.7%		
	MAC-T	ssODN	34%		
	MAC-T	ssODN	19.6%	8.33%	0.00%
	Fetal Fibro	ssODN	7.7%		
	Fetal Fibro	ssODN	11%		
4	MAC-T	ssODN	15.6%	5.21%	0.00%
	MAC-T	ssODN	12.9%	13.54%	15.38%
	MAC-T	ssODN	5.1%	15.63%	13.33%
5	Fetal Fibro	ssODN	8.2%	3.13%	0.00%
	Fetal Fibro	ssODN	8.2%	5.21%	20.00%

2.4 Discussion

The introduction of engineered nucleases has enhanced scientist's ability to create edits in organism's genomes. The CRISPR/Cas9 system has been widely used in mice and a variety of cell lines but has not yet been widely used in livestock species. Very few experiments have attempted to use the Cas9 nuclease to make edits to the bovine genome. In this experiment, we attempted to knock-in a point mutation using homology directed repair (HDR), into MAC-T cells, a bovine mammary epithelial cell line (15). This change would allow us to see the effects of the mutation at the (+15) position of the alpha lactalbumin gene sequence. In this experiment, we did not make the desired single nucleotide change via HDR in MAC-T cells, but we did create indels at the desired location. Although we did not induce HDR in MAC-T cells, we were able to induce a knock-in in bovine fetal fibroblasts using the Cas9 system paired with a ssODN.

Through this experiment we found that this specific line of MAC-T cells are heterozygous for the mutation at the (+15) position. Other studies have used MAC-T cells to determine if CRISPR/Cas9 system can be used to disrupt genes in bovine cells (18). While we were not able to make a cell line that was homozygous for the adenine mutation, we did create a 20 nucleotide knock-out in the α-LA sequence which included the transcriptional and translational start point, which could change the production of milk proteins in these cell lines. Originally, MAC-T cells were transfected with pSpCas9(BB)-2A-Puro and selected using puromycin selection, which resulted in large cell death and it was difficult to recover colonies after selection. By switching to pSpCas9(BB)-2A-GFP and using flow sorting to select colonies, we were able to analyze the cutting efficiency of the CRISPR system. MAC-T cell DNA was analyzed using enzyme digestion with BpuEI, an enzyme that created a cut in the PCR product if the mutation was present; when a deletion of the adenine at α-LA (+15) occurred, a single band

was seen on a gel. If there was a single band the DNA was sent to sequencing, this confirmed that the CRISPR was cutting in the desired region and induced some level of gene editing.

Once we confirmed that the CRISPR system targeted the desired region in MAC-T cells, we applied the system to bovine fetal fibroblasts. The CRISPR system has been shown to work in fetal fibroblasts, there have been fetal fibroblasts with knock-outs (19) and knock-ins (18) in fetal fibroblasts. Edited fetal fibroblasts can be used for nuclear transfer, which is optimal because once you have an edited cell line you can be certain that the embryo will contain the desired edit. To create a single nucleotide change a 90 bp ssODN was combined with Cas9 DNA. In order to detect the mutation, DNA was extracted from cells and then digested with BpuEI. Although we saw low survival of fetal fibroblasts after sorting, we were still able to detect a colony with single base pair change using a ssODN.

The inability to create a knock-in in MAC-T cells may be due to low survival rate. Low survival rate of MAC-T cells and fetal fibroblasts can be attributed to a variety of issues. One issue may be that cells grow more efficiently when in groups. Cell sorting places five cells per well but sometimes the stress is too great and they do not attach and grow. When the CRISPR system was discovered and scientist began to use it to make edits to organisms genomes, plasmid DNA was the main method of delivering the Cas9 nuclease (14). As the system was developed studies showed that plasmid DNA is more stressful for a cell then Cas9 protein or mRNA (20). Plasmid DNA can be stressful to the cell because it triggers cyclic GMP-AMP synthase activation (21). It also has an increased potential for off target effect, this may be due to the fact that it can be expressed in a cell for several days (22), while protein and mRNA have a fast turnover rate which may attribute to their reduced off-target effect (23). Another issue could be off-targets, which may have negative effects on the cell and decrease the survival rate.

Transfection reagents also play a role in cell survival. For this experiment, we originally used FuGENE® 6 as a transfection reagent and saw about 30% of cells fluoresced when transfected with eGFP plasmid. This was surprisingly low based on other papers that showed FuGENE® 6 resulted in greater survival and a high transfection rate (88%) compared to Lipofectamine® 2000 (24). Because we were working with MAC-T cells we also looked at the efficiency of electroporation and Lipofectamine® 2000, as seen in the paper above, very few colonies survived those methods. One study recently looked at 60 different transfection reagents used to deliver Cas9 protein in a variety of cell lines. They saw that Lipofectamine™ CRISPRMAX™ worked in most cell lines but in MCF-7 cells (human mammary gland) they saw very low percent indels formed with all transfection methods, Lipofectamine™ CRISPRMAX™ only resulted in 8% indel formation (25). These results lead me to believe that since MAC-T cells are a mammary epithelial cell line, and other mammary cell lines are difficult to transfect, that MAC-T cells may also be difficult.

While we were not able to knock-in the ssODN into MAC-T cells, we were successful in fetal fibroblasts. A variety of studies have shown that bovine fibroblasts can be edited using CRISPR/Cas9 system and then used for nuclear transfer (18,19,26). These studies used different methods to deliver Cas9 and saw varying results. One study used electroporation in combination with linearized plasmid DNA repair template; sgRNA, Cas9 nuclease, and reporter plasmid were all delivered separately to insert 2.08 kb knock-in vector of human fibroblast growth factor 2. They saw that CRISPR/Cas9 system was able to induce HDR in bovine primary fibroblast with a high efficiency (75.4%-80%) (18). Using SCNT they saw 25.7% of KI FGF cells reached the blastocyst stage at day 8, this was the first time CRISPR/Cas9-mediated homologous recombination was shown in bovine fibroblasts(18). Another experiment used cytoplasmic

injection of either plasmid coding for Cas9 or mRNA encoding Cas9, and higher blastocyst rates were achieved using RNA. For their HDR plasmid they targeted an 875 bp sequence on PRNP exon 3 with 1 Kb homologous arms flanking the region, with an EGFP region. Mutations were detected in 25%-45% of the blastocysts evaluated, indels were seen only in groups treated with RNA but not DNA, and HDR occurred in one out of eight embryos treated with RNA (26). In our experiment, we used plasmid DNA that had the sgRNA inserted into the gRNA scaffold of pSpCas9(BB)-2A-GFP, this was combined with a 90 bp ssODN. This is the first study to report the use a ssODN to make a single nucleotide change in fetal fibroblasts to our knowledge.

Numerous diseases and mutations are linked to a single nucleotide polymorphism (SNP) that has been identified by genetic studies. Donor plasmids are often used to create genomic modifications, these plasmids contain large homology arms and can take weeks to design and require skill (27). A study used ssODN in combination with ZFN to make a single nucleotide change and showed that ssODN are cheap and easy to design and can efficiently create a SNP (28). This strategy was then used in combination with CRISPRs instead of ZFN in porcine fetal fibroblasts, where they saw 10% of the cells contained the desired change, many of which were only heterozygous for the change. 930 embryos were made using SCNT and placed in four recipients, only one pregnancy came to term and it was a still born (29).

In conclusion, ssODN can be used to make a single nucleotide change in fetal fibroblasts. Other studies have shown that edited fetal fibroblast can then be used in combination with SNCT to create edited embryos. This holds great promise for the future of curing diseases or inserting beneficial mutations to improve livestock production.

In future studies, based on the frequency of ssODN repair in fetal fibroblasts, it is likely that if a larger number of MAC-T cell colonies were screened, a knock-in might be detected.

These cells could then be used to understand the function of the adenine mutation at the (+15) in the 5' flanking region of the α-LA transcriptional start region. The use of mRNA or protein instead of plasmid DNA may increase editing efficiency and survival rate. Also, future experiments could use fetal fibroblasts, that have a single nucleotide change, for nuclear transfer to create an edited embryo.

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Chapter 3

Introducing a Single Nucleotide Change in Bovine Embryos Using Sperm-Mediated Gene Transfer

Abstract

Microinjection is the most common gene transfer method and works at a high efficiency in mice but similar results are not seen in livestock. Naked DNA has been shown to bind naturally to the sperm, this method is called sperm-mediated gene transfer (SMGT) and has been demonstrated in pigs (1) and cattle (2). Based on these observations, we examined the efficiency of exogenous DNA binding to sperm using liposomes. In this experiment, we analyzed methods to select thawed bovine sperm, and evaluated the binding of exogenous DNA to those sperm. To determine the optimal sperm selection method, sperm was analyzed using a Computer-Assisted Sperm Analysis (CASA), the parameters selected were: total motility (TM), rapid motility (RM) and progressive motility (PM). Liposome preparation was done using the cationic lipid, 3-(trimethyl ammonium iodide) 1,2 dimystryl-propanediate (TAID) and a neutral lipid, L-α Dioleoyl phosphatidyl-ethanolamine (DOPE) prepared according to the protocol (3). Percoll gradients or swim-up methods were used to select sperm after thawing (4), followed by incubation (1h or 3h) with the liposome-DNA complexes according to the liposome preparation protocol (3). We used enhanced green fluorescent protein (eGFP) in combination with the liposomes as a marker for exogenous DNA binding. Five treatments per selection method were analyzed: 1) no incubation, no liposomes and no DNA, 2) incubation with no liposomes and no DNA, 3) incubation with liposomes and no DNA, 4) incubation with liposomes and 1 ng of DNA and 5) incubation with liposomes and 10 ng of DNA. Once the liposomes had been complexed with DNA they were incubated with sperm for one or three hours before IVF. This was repeated five times. The CASA results for total motility and rapid motility were significantly ($P<0.01$)

between the control and the other treatments in the Percoll group as opposed to swim-up. Swim up was therefore chosen as the optimal selection method. In order to determine if the liposome-DNA complex had bound to sperm, real time PCR was used to detect GFP DNA and images of the sperm were analyzed using the Spatial Light Interference Microsocopy (SLIM). SLIM confirmed the presence of liposomes on the sperm head and tail. SMGT was then used with *in vitro* fertilization (IVF) to deliver plasmid GFP and the CRISPR to make edited embryos. Blastocyst rate was low and ranged from 0 to 20%. None of the 8 day embryos tested positive for GFP.

3.1 Introduction

As people move out of the poverty level there is an increase in consumption of meat and milk (5). In 2050, the population is expected in increase to ~9 billion people and the majority of that growth will occur in developing countries. One way to meet the food demand is by genetically modifying animals. In mice, microinjection is the most common method used for gene transfer to produce transgenic animals. While this method can be used in livestock it requires much skill, time and expense, unlike sperm-mediated gene transfer (SMGT), which requires less skill (6). Sperm-mediated gene transfer has been successfully used to transfer DNA to embryos using the sperm's ability to bind naked DNA (7-9). In this case sperm is used as the vector for specific gene transfer, meaning the transportation of exogenous DNA into the oocyte during fertilization. SMGT has been reported in mice (8,10-12), cows (2,9,13), and other species.

Augmentation techniques are also used with SMGT, these include methods that use electroporation or liposomes to drive the sperm to take up the transgene DNA. Liposome/DNA delivery methods are another technique under study for introducing

exogenous DNA into cells and embryos. Liposomes are small structures consisting of membrane-like lipid layers or bilayers, which can actually protect foreign DNA from digestion by proteases and DNAse (14). Cationic liposomes are capable of spontaneously interacting with DNA molecules, giving rise to lipid-DNA complexes (15). It is proposed that ionic interactions between the positively charged hydrophilic exterior of liposomes and the negatively charged phosphate groups of DNA molecules are responsible for the lipid-DNA complex formation (16). Delivery of foreign DNA into the target cell occurs via fusion of the lipid-DNA complex with the cell membranes. Under appropriate conditions, exogenous DNA can be transferred into cells and a portion of this DNA becomes localized in the nucleus (15).

Both transient and stable transformation of mammalian cells has been widely shown in the literature. Liposomes generally result in higher transfection efficiencies as compared to electroporation or calcium precipitation (15). An advantage of cationic liposomes is that the DNA size does not seem to be a limiting factor during transfection (17,18). Liposome-mediated gene transfer has been shown to be a highly efficient method for transforming cultured cells with foreign DNA. Liposomes have also been a very effective tool to transfect spermatozoa (19). The major advantage to sperm-mediated transfer for producing transgenic animals is its simplicity; however, the disadvantage of this technique is the decreased ability of the host's genome to incorporate DNA presented, and the instability of replicating previous experiments.

Cationic lipids combined with neutral lipids were to enhance binding of DNA to sperm. This was then used for SMGT with fresh boar semen for AI in pigs. 94% of day 8 embryos contained the GFP plasmid that had been complexed with liposomes for SMGT (3). In the

present experiment, we analyzed methods to select thawed bovine sperm for DNA binding, and evaluated the binding of exogenous DNA to those sperm. Sperm was evaluated using a Computer-Assisted Sperm Analysis (CASA), qPCR and SLIM imaging. In the subsequent experiment, SMGT was then used to create embryos. *In vitro* fertilization (IVF) was performed with sperm that had been incubated with liposome-DNA complex. Seven days after fertilization (day 8 of development), embryos were evaluated and DNA was extracted. The average blastocyst rate was ~6% and none of them were GFP positive.

3.2 Materials and Methods

Sperm Selection Methods

Semen straws were removed from the liquid nitrogen tank and thawed 37°C for 40 seconds. After thawing, the semen was processed with two standard protocols: swim-up or Percoll discontinuous gradients (45% to 80%). Percoll discontinuous gradients were prepared by combining Sperm-TALP and ISO-Percoll. Sperm-TALP- basic- medium (20), was supplemented with pyruvic acid (Sigma-Aldrich, St. Louis, MO). and gentamycin sulfate (Sigma-Aldrich, St. Louis, MO). ISO-Percoll was made by combining sodium bicarbonate with Earle's Balanced Salt Solution (EBSS) (Thermo Fisher Scientific, Waltham, MA) and vortexed in a tube, which was then combined with Percoll® (Sigma-Aldrich, St. Louis, MO). The pH was adjusted to 7.4 and the solution was filtered through a .22 μm filter. ISO-Percoll and Sperm-TALP were combined to create 45% and 80% mixture of Percoll. 80% Percoll was first added to the tube then 45% Percoll was slowly added as a second layer.

Semen was prepared for discontinuous gradients by pooling three straws of frozen-thawed bull semen. The semen straws were removed from the liquid nitrogen storage tank and immediately placed in warm water at 37°C for 40 seconds. Semen was then slowly pipetted down the side of the tube to create a third layer on top of the 45% ISO-Percoll and then centrifuged at 460 x g for 25 minutes. The supernatant was discarded and the pellet was washed in Sperm-TALP and centrifuged at 250 X g for 10 minutes. This was repeated a third time, removing the supernatant and suspending the pellet in Sperm-TALP and centrifuging it at 170 x g for 10 minutes. The supernatant was removed and 50 µl of Sperm-TALP was added to the pellet. The sperm was then either evaluated by CASA or was incubated with liposome/ DNA complexes.

Swim-up

The sample of thawed semen was layered carefully under 1 ml of equilibrated sperm-TALP (Tyrode's albumin lactate pyruvate) medium with 6 mg of BSA per ml in a centrifuge tube. After loading, the tube was placed in incubator at 39 °C for 1 hour. After incubation, 400 µL of the upper fraction of TALP (containing the selected sperm) was collected, placed in a tube and centrifuged for 10 min at 160 x g (21). The obtained pellet was then used for either qPCR to evaluate the presence of DNA attached to sperm or IVF.

Liposome Preparation

The cationic lipid, 3-(trimethyl ammonium iodide) 1,2 dimystryl-propanediate (TAID) was synthesized by Russell (3) and used for the subsequent studies. The neutral lipid, L-α Dioleoyl phosphatidyl-ethanolamine (DOPE) was purchased from Sigma-Aldrich (Sigma-

Aldrich, St. Louis, MO) and used without further purification. DOPE was reconstituted in a 9:1 ration of $CHCl_3$:MeOH. Once mixed, the TAID and DOPE were dissolved in the organic solvent chloroform ($CHCl_3$; Sigma Aldrich, St. Louis, MO) at a molar ratio of 2:1 in a round bottom flask. A rotary evaporator was then used to concentrate the lipid at the bottom of the flask at 40-50°C. When the chloroform was completely evaporated, three μl of TALP were added to the round bottom flask containing the lipid film. A cell scraper was then used to scrape the layer of lipids from the glass surface, then the sample was pipetted up and down for one minute followed by a minute of vortexing. The flask was placed in an incubator at 37°C for 15 minutes in a 5% CO_2 in air atmosphere. Fifty (50) μl of the liposome-TALP mix were added to tubes containing zero, one, or ten micrograms of DNA. Liposome/DNA complexes were incubated at 37°C for 15 minutes at 5% CO_2. Collected sperm were added to medium alone (control) or medium containing liposome/ DNA complexes. The 50 μl were then added to 150 μl of TALP and sperm. The final concentration of the solution is 150 μM lipids, 1 or 10 μg DNA and sperm in TALP. This mixture was placed at 37°C in 5% CO_2 for three hours. Following incubation, 3 μl of the sample was analyzed by CASA and the rest was placed in the -80°C freezer to be saved for DNA extraction.

Computer-assisted semen analysis, CASA IVOS (Hamilton Thorne Bioscience, Beverly, MA) system was used to measure total motility, rapid motility and progressive motility of sperm. The software was set per the manufacturer's recommendations for the assessment of motility characteristics of bull spermatozoa. According to the recommendations of the manufacturer the equipment was set as followed; frames acquired: 30; frame rate: 60 Hz/s; minimum contrast for cell detection: 80; minimum cell size: 5 pix; Progressive VAP 50 μ/s; straightness threshold: 70%; slow VAP cut off: 30 μ/s; slow VSL cutoff: 15 μ/s; and magnification factor 1.92. For each

sample 3 µl was removed and ten microscopic fields were analyzed. The image knob was adjusted to make the image clear on the monitor.

Sperm Genomic DNA Extraction

Samples frozen at -80°C were thawed at room temperature. Samples were centrifuged at 15,600 x g for five minutes and the supernatant was removed. Five hundred (500) µl of 70% ethanol was added to the pellet and centrifuged again at 15,600 x g for five minutes. Cells were lysed by adding 500 µl of lysis buffer, which was composed of 2 ml of 5 M NaCl, 1 ml of 1M tris, 2.5 ml of 1M EDTA (Thermo Fisher Scientific, Waltham, MA), 5 ml 10% SDS, and 89.5 ml ddH_2O for a final volume of 100 ml. Then 2.5 µl of 0.5% Triton® X-100, 21 µl of 1M dithiothreitol (DTT), and 40 µl of 10 mg/µl of proteinase K (Invitrogen- Thermo Fisher Scientific, Waltham, MA) were added to the sample. Samples were then vortexed and incubated at 50°C overnight on a shaker with moderate shaking. The following day the samples were centrifuged for 10 minutes at 15,600 x g and the supernatant was transferred to a new 1.5 ml tube. In order to precipitate the DNA, 1 µl of 20 mg/ml glycogen and 1/10 volume of 3M NaAc was added to the supernatant. Then two volumes of ice-cold absolute ethanol was added and placed in -80°C for 1-2 hours. The DNA was then pelleted by centrifuging the sample for 20 minutes at 15,600 x g. The supernatant was removed and the pellet was then washed with 500 µl of 75% ethanol and centrifuged for 10 minutes at 15,600 x g. Ethanol was pipetted slowly from the tube and then the tube was left at room temperature until the remaining ethanol had evaporated. The DNA was dissolved in 30 µl of TE buffer, which was composed of 10 ml of 1 M TRIS pH 8.0, 1 ml of 1M EDTA, and brought to a final volume of 1000 mL with ddH_2O. The DNA was left at 4°C overnight and the concentration was measured the next day using a

NanoDrop 1000 Spectrophotometer nucleic acid analyzer (Thermo Fisher Scientific, Waltham, MA). The DNA samples were stored at -20°C.

Quantitative Real-time PCR (qRT-PCR) Analysis

qPCR was performed using SYBR® Select Master Mix for CFX (Thermo Fisher Scientific, Waltham, MA). A total volume of 10 µl contained 6 µl of SYBR select mix, 0.4 µl of forward and reverse primer, 4 µl of gDNA and 0.2 µl of ddH2O. The thermo cycling profile was the following; stage 1: 50°C for 2 min, stage 2: 95°C for 10 min, stage 3: 95°C for 15 sec then 65°C for 1 min repeated for 40 cycles, stage 4: 95°C for 15 sec then 60°C for 15 sec. Three replicates were carried out for quantification of the target gene. Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ) was amplified for each sample as a housekeeping gene for bovine sperm and GFP was used as a primer to amplify GFP plasmid. Primers to detect eGFP were (CATGGTCCTGCTGGAGTTCGTG) and (CGTCGCCGTCCAGCTCGACCAG). The control primes for YWHAZ used were forward (GCATCCCACAGACTATTTCC) and reverse primer (GCAAAGACAATGACAGACCA).

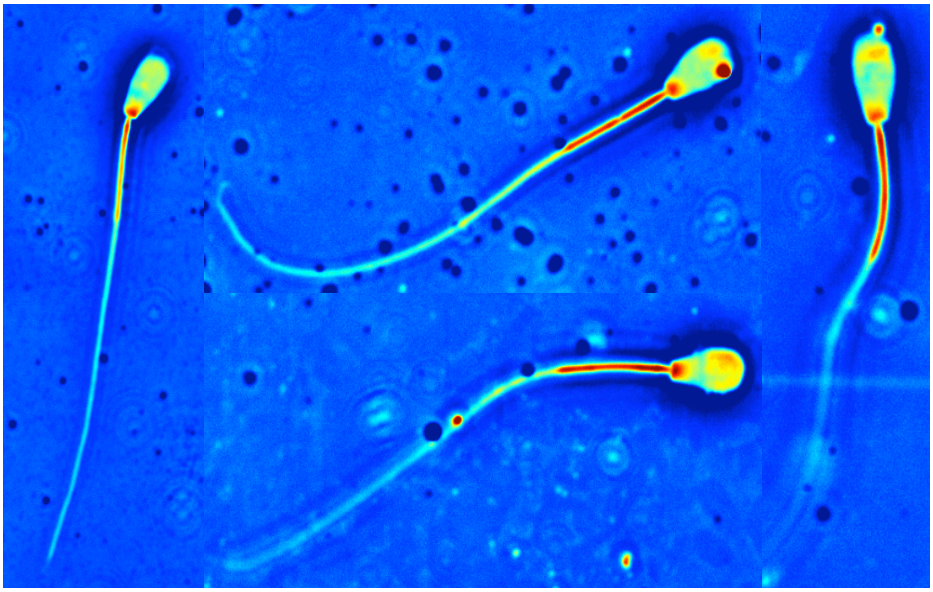
SLIM Analysis

Sperm was removed from 1.5 ml tube after being incubated with liposome mixture for one or three hours before it was used for IVF or other sperm analysis. Ten (10) µl of sperm was added to a glass slide, the sample was drawn across the slide to spread it evenly. These slides were air dried and stored at 4°C until ready for analysis. Pictures were taken of the slides using the Spatial Light Interference Microsocopy (SLIM) at the Beckman Institute in the laboratory of Dr. Gabriel Popescu, this method uses phase contrast microscopy and holography to evaluate

multiple light waves which allows the visualization of nanoscale structures without staining. SLIM was used to detect the presence of liposomes on the sperm head and tail (Figure 15). SLIM depicts the density of the image, liposomes attached to sperm head or tail showed had greater density than the surrounding area, these areas of greater density were captured and then the diameter of the liposome was measured. Liposomes were measured using jet color map on imageJ, a line was drawn across the liposome to measure its density. This gave the number of pixels, which was then divided by 7.14 to give the value of the diameter in μm.

Figure 15: SLIM Images

Images were obtained using jet color map on imageJ with SLIM microscope. Liposomes appeared as red spots on the head and tail because those regions had a higher density. Image on the left is sperm with no liposome attaches, the other three have liposomes attached.



Embryo Production

Jersey bull semen was obtained from the University of Illinois Urbana-Champaign Dairy Farm, samples were tested using enzyme digestion to confirm that they did not have an adenine in the (+15) position in the 5' flanking region of the α-LA sequence. Liposomes were prepared

as stated previously, 1 μg of DNA was added to 50 μl of liposome-TALP. Sperm was thawed and selected using swim-up. Sperm were counted and then diluted to have a final concentration is $1x10^6$ sperm/ml. One fourth of the final volume was removed from 50 μl liposome DNA mixture and added the sperm TALP mixture. For example, if the sperm was in 30 μl of TALP, 10 μl of liposome mixture was added. The sperm and liposome mixture were incubated for one or three hours at 37°C in 5% CO_2. There were five different experimental groups; group one had no liposome and no DNA, group two had liposome and no DNA, group three had liposome and 1 μg of eGFP plasmid DNA, group four contained liposome and 1 μg of pSpCas9(BB)-2A-GFP DNA, and group five had 0.5 μg of pSpCas9(BB)-2A-GFP DNA and 0.5 μg of ssODN. After the sperm and liposome mixture were incubated for one or three hours they were added to the oocytes.

One set of oocytes was purchased from DeSoto Biosciences, Seymour, TN and delivered in culture medium, they were kept in *in vitro* maturation for 24 hours. The second set of oocytes were aspirated from ovaries that were obtained from a slaughterhouse and transported to the lab in physiological saline at 30–35 °C. Cumulus–oocyte complexes (COCs) were aspirated from follicles of 2–8 mm in diameter. Oocytes with uniform cytoplasm and multilayered cumulus cells were selected, washed twice in HEPES-TCM medium (TCM 199 supplemented with 25 mM of HEPES, 2 mM sodium bicarbonate, 2 mM sodium pyruvate, 1 mML -glutamine, 10 μl⁄ml amphotericin B) supplemented with 95.6 USP⁄ml heparin and then transferred in the IVM medium, that was TCM 199 supplemented with 15% bovine serum (BS), 0.5 μg⁄ml FSH, 5 μg ⁄ml LH, 0.8 mM L -glutamine and 50 μg⁄ml gentamycin. Groups of 25 COCs were matured in of IVM medium, covered with mineral oil, in four well plates, for 22–24 h at 39 C, 5% CO_2 in air (20).

Treated sperm was added to wells containing 30-50 oocytes that were in IVF medium, Tyrode's modified medium (22) without glucose and bovine serum albumin (BSA), and supplemented with 5.3 SI/mL heparin, 30 μM penicillamine, 15 μM hypotaurine, 1 μM epinephrine and 1% of BS. Gametes were co-incubated for 20 h at 39°C, in 5% CO_2 in air, after which presumptive zygotes were vortexed for 2 min and then pipetted to remove cumulus cells in HEPES-TCM with 5% BS, then they were washed twice in the same medium and transferred 30–50 zygotes per well into 400 μl Synthetic Oviduct Fluid (SOF) medium (23), supplemented with 30 μl/ml essential amino acids, 10 μl/ml non-essential amino acids, 0.34 mM tri-sodium citrate, 2.77 mM myo-inositol and 5% BS. Zygotes were incubated in a humidified mixture of 5% CO_2, 7% O_2 and 88% N_2 at the temperature of 39°C.

Embryo Analysis

Embryos were placed into 10 μl of PBS, frozen, and stored individually at −80°C until they were assayed. Samples were removed and thawed at room temperature, genomic DNA was extracted by adding twenty microliters of lysis buffer containing 15 mM Tris–HCl pH 8.9, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) and 150 μg/ml proteinase K (Sigma-Aldrich, St. Louis, MO) to each tube. The tubes were incubated at 55°C for 1 h and then at 90°C for 10 min to inactivate proteinase K. DNA was then evaluated using PCR with JumpStart™ REDTaq® DNA Polymerase (Sigma-Aldrich, St. Louis, MO) with primers for GFP and control primers btRep-137 C1 and btRep-137 C2. The control primers are btRep-137 C1 (TATTTTCGGAACGCGGGAGAGAAGAG) and btRep-137 C2 (TATTTTTGATTCCCTCCGTGCGGCGCTTA) which result in 450 bp, the primers for GFP are forward primer (ACCTACGGCGTGCAGTGCTT) and reverse primer (AGGACCATGTGATCGCGCTT) resulting in 467 bp product. The GFP primers amplified the

GFP sequence in both pIRES2-EGFP and pSpCas9(BB)-2A-GFP plasmids. PCR cycling parameters were: 1 min at 94°C, 30 sec at 94°C, 30 sec at 60 °C, 1 min at 72 °C, go to step 2 and repeat 30 times, 1 min 72°C, and 5 min 4°C. Samples were then size-separated on 2% agarose gels.

Statistical Analysis

The results for CASA were analyzed as the mean rapid, progressive or total motility for five different groups in five replicates. The statistical differences between them were analyzed using a *Chi* square test with Yates correction was performed using Excel. Differences were considered to be significant for $P < 0.05$. A *Chi* square test with Yates correction was also used to evaluate qPCR data. There was no significant difference between one hour and three hours of incubation based on CASA results, using generalized least squares (GLS) ANOVA analysis. Therefore, the average percent motility of the different groups for one hour and three hours were combined to determine the differences between the methods used. These were also analyzed using GLS ANOVA. SPSS version 19 (IBM, New York) was used to evaluate the differences between groups based on the percent of liposomes attached to sperm using Post Hoc analysis with Bonferonni correction.

3.4 Results

Sperm incubation with Plasmid GFP

Bovine sperm was combined with liposomes and DNA to be used for SMGT. The motility of the sperm was measured to determine the optimal method to attach DNA to sperm for SMGT. Two different sperm selection methods were used, swim-up and Percoll gradient. For each of these methods zero, one, or ten micrograms of DNA were added to liposomes. Sperm

motility with no liposome and no DNA after 1 or 3 hours of incubation was measured as a control. The liposomes were then incubated with DNA and combined with sperm to be incubated for one to three hours.

The sperm was analyzed using CASA to measure total motility, rapid motility and progressive motility of sperm. The average total motility, progressive motility and rapid motility of sperm using Percoll and swim-up selection after one hour are seen in Table 2.A, and after three hours in 2.B. There was no significant difference when we evaluated the time variable using generalized least squares (GLS) ANOVA. Therefore, the average percent motility of the different groups for one hour and three hours were combined to determine the differences between the methods used. These were also analyzed using GLS ANOVA (Figure 16). The percentage of total motility (TM), progressive motility (PM), and rapid motility (RM) for Percoll were significantly decreased in sperm with liposomes compared to the control ($P<0.05$). When using swim-up selection, a difference between the control group can be seen in the group where 10 μg of DNA is combined with liposomes but not when 1 μg of DNA is added for TM, PM and RM (Figure 16).

Table 2: CASA Results

A. The average motility (± SE) for each group with Percoll and swim-up selection after 1 hour. The first control is the motility of the sperm immediately after selection.

Method		Total Motility	Progressive Motility	Rapid motility
	Experiment	**Average**		
Percoll	**Control (T0)**	87.50 (2.67)	73 (0)	82.30 (2.67)
	No liposome	41.50 (26.75)	26.50 (19.94)	30.00 (20.51)
	Liposome no DNA	26.25 (3.20)	14.75 (2.50)	18.00 (3.56)
	Liposome 1 mg DNA	38.00 (17.03)	20.25 (9.07)	25.25 (12.58)
	Liposome 10 mg DNA	24.50 (4.43)	13.50 (2.65)	15.00 (3.37)
Swim-up	**Control (T0)**	61.00 (31.10)	44.75 (20.88)	51.50 (25.077)
	No liposome	77.25 (9.18)	53.00 (7.26)	59.50 (3.87)
	Liposome no DNA	68.50 (11.70)	49.50 (11.70)	51.50 (10.41)
	Liposome 1 mg DNA	60.75 (31.32)	49.00 (26.39)	54.00 (30.11)
	Liposome 10 mg DNA	56.75 (17.29)	33.50 (14.84)	37.75 (14.50)

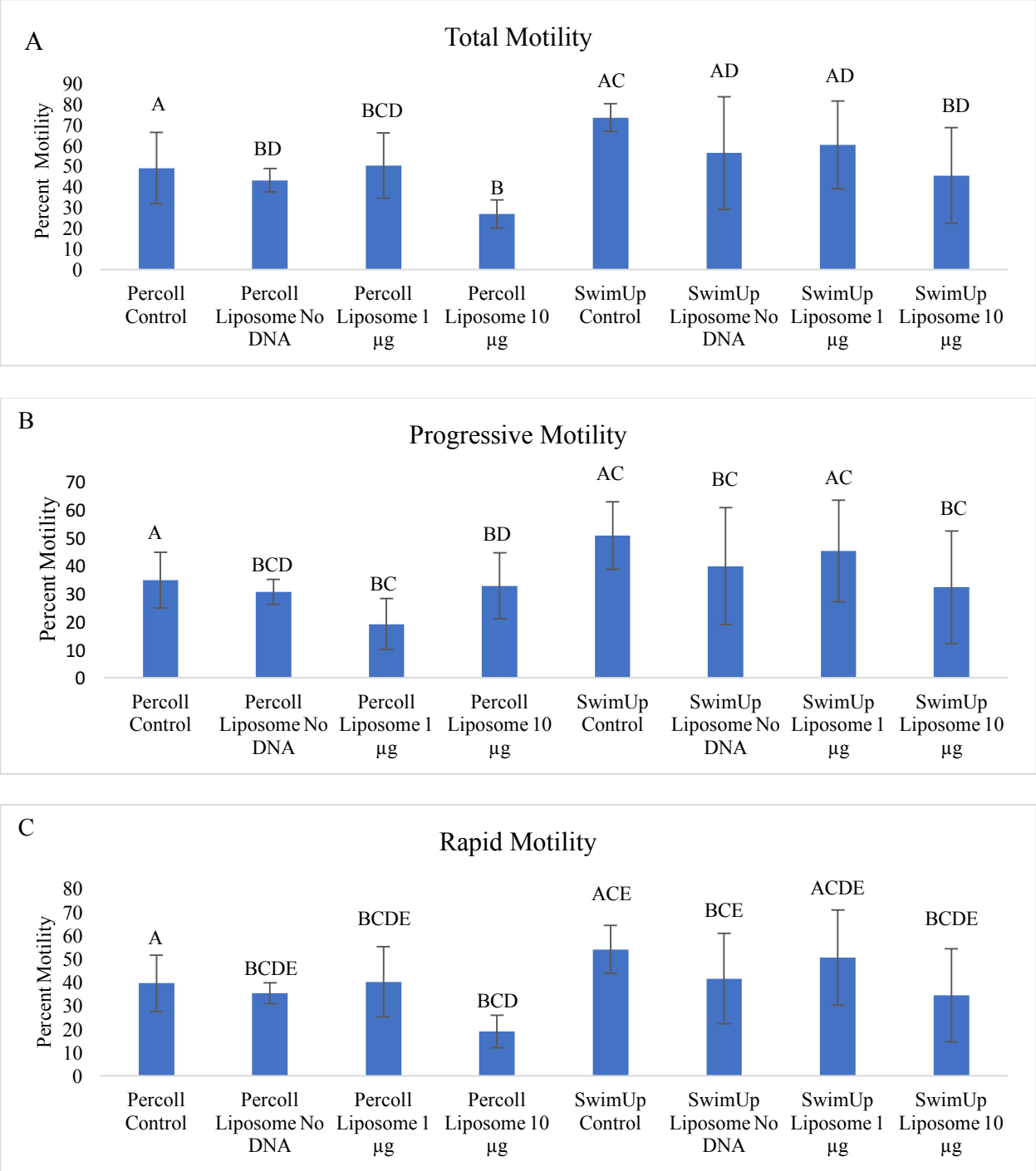
Table 2: CASA Results (cont.)

B. The average motility (± SE) for each group with Percoll and swim-up selection after 3 hours. The first control is the motility of the sperm immediately after selection.

Method		Total Motility	Progressive Motility	Rapid motility
	Experiment	Average		
Percoll	Control (T0)	87.50 (2.67)	73 (0)	82.30 (2.67)
	No liposome	56.50 (7.50)	43.00 (0)	49.00 (3.46)
	Liposome no DNA	60.00 (8.08)	46.50 (6.35)	52.50 (5.20)
	Liposome 1 mg DNA	62.50 (14.43)	18.00 (9.24)	55.00 (17.32)
	Liposome 10 mg DNA	29.00 (9.24)	52.00 (20.78)	23.00 (10.39)
Swim-up	Control (T0)	61.00 (31.10)	44.75 (20.88)	51.50 (25.077)
	No liposome	69.50 (4.04)	48.50 (16.74)	48.50 (16.74)
	Liposome no DNA	44.00 (42.72)	30.00 (30.02)	31.50 (28.29)
	Liposome 1 mg DNA	59.50 (10.97)	41.50 (9.81)	47.00 (10.39)
	Liposome 10 mg DNA	34.00 (28.87)	31.00 (25.40)	31.00 (25.40)

Figure 16: CASA results without time comparison

Comparison of CASA results for motility with standard deviation. The average motility of sperm that was incubated for 1 and 3 hours was combined to exclude the timing factor.



[ABCDE]Least square means (± SE) within each column with different superscripts differ (P<0.05).

To determine if plasmid DNA had attached to the sperm, genomic DNA was extracted and then analyzed by qPCR. Five sets of triplicates were quantified for each group, resulting in a total of 15 samples. In order to determine the efficiency of plasmid eGFP attaching to sperm, we looked at Ct (threshold) values for eGFP and YWHAZ were compared. A number one was given to each positive Ct value for the primers, the one values were added together for each treatment and then eGFP was divided by YWHAZ to show what percent of DNA had bound to sperm. For samples where no eGFP DNA was added, there were no positive Ct values. 66.67% of sperm selected using Percoll and incubated with liposomes 1 μg of DNA had positive Ct values, 100% with 10 μg of DNA had positive Ct values. Sperm selected using swim-up had 57.14% of eGFP DNA attach with 1 μg of DNA and 100% with 10 μg of DNA (Table 3). These values were compared using a *Chi* square test with Yates correction. No difference was seen between the swim-up and Percoll selection methods when the same amount of DNA was added. A difference was seen in attachment of DNA between groups that had 0, 1 or 10 μg of DNA added (Table 4).

Table 3: qPCR Results

qPCR products that had positive Ct values for pEGFP and YWHAZ. Methods used to select sperm were P- Percoll, S- swim-up. DNA added to sperm was 0,1, and 10 μg.

Gene	**P0**	**P1**	**P10**	**S0**	**S1**	**S10**
GFP/YWHAZ %	0	66.67	100	0	57.14	100

Key		
	Method	**μg DNA**
P0	Percoll	0
P1	Percoll	1
P10	Percoll	10
S0	Swim-up	0
S1	Swim-up	1
S10	Swim-up	10

Table 4: Comparison of qPCR data

Based on the percent of GFP percent divided by control gene YWHAZ a percent was given. For each group the percentage was compared using *Chi* square analysis.

	P0	**P1**	**P10**	**S0**	**S1**	**S10**
P0	Not sig	1%	1%	Not sig	1%	1%
P1	1%	Not sig	1%	1%	Not sig	1%
P10	1%	1%	Not sig	1%	1%	Not sig
S0	Not sig	1%	1%	Not sig	1%	1%
S1	1%	Not sig	1%	1%	Not sig	1%
S10	1%	1%	Not sig	1%	1%	Not sig

Liposome Verification with SLIM Microscope

After the sperm had been incubated for one or three hours a drop of liposome-sperm complex was placed on a glass slide and then air dried to be evaluated using SLIM microscope (Table 5). The average size of a liposome can range from small (0.025 μm) to large (2.5 μm) sized vesicles (24). The size of liposomes did not vary significantly between groups and the average size was as expected (Table 5). SPSS version 19 (IBM, New York) was used to evaluate the differences between groups based on the percent of liposomes attached to sperm using post hoc analysis with Bonferonni correction. There was a significant difference between time point zero and one hour, and time zero and three hours but not between one and three hours. This was determined by eliminating groups and evaluating time only to determine if the microscope can detect liposomes. We see that 1 and 3 hours are different than time point zero (Figure 17). The average percent of liposomes bound to sperm varied between groups (Table 6). This variation was also analyzed using the Bonferroni correction. After one hour of incubation there is a difference between Percoll control and the groups with liposomes except for Percoll and 1 μg of DNA, and the same was seen in swim-up. After three hours of incubation the percent of

liposomes bound was different between Percoll control and the other Percoll groups with liposomes, but it was only different the swim-up when 10 µg of DNA was added (Figure 18).

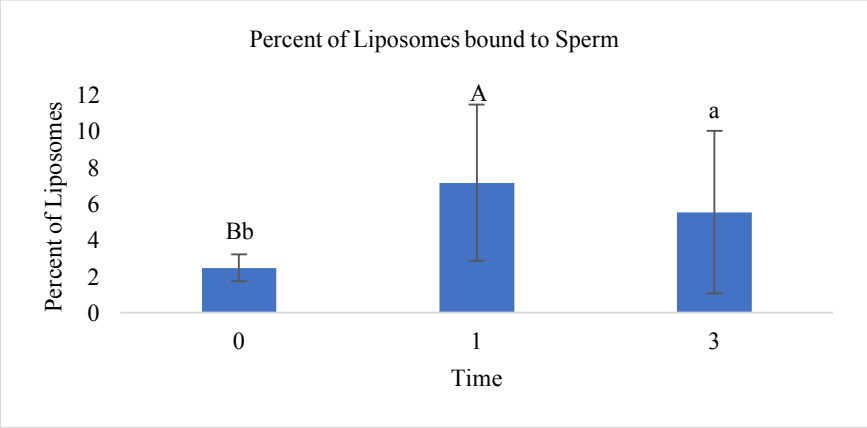
Table 5: Average size of liposomes

The average liposome size in µm (± SE) found on sperm head or tail using SLIM imaging.

	Average Size (µm)								
	0 hour	**1 hour**				**3 hour**			
	Control	**Control**	**Liposome**	**1**	**10**	**Control**	**Liposome**	**1**	**10**
Swim-up	1.94 (0.60)	1.83 (0.46)	1.74 (0.67)	1.82 (0.58)	1.84 (0.64)	1.77 (0.56)	2.35 (1.17)	2.12 (0.89)	1.92 (0.64)
Percoll	1.98 (0.46)	2.38 (0.90)	1.69 (0.49)	2.11 (0.72)	1.83 (0.63)	1.69 (0.72)	1.60 (0.71)	2.03 (0.81)	2.20 (0.66)

Figure 17: Evaluation of percent of liposomes bound to sperm based on time

Comparison of the percent of liposomes bound to sperm between time 0,1 and 3 without group effect. Averages for each group were combined.



[AB]Least square means (± SE) within each column with different superscripts differ (P<0.01).
[ab]Least square means (± SE) within each column with different superscripts differ (P<0.05).

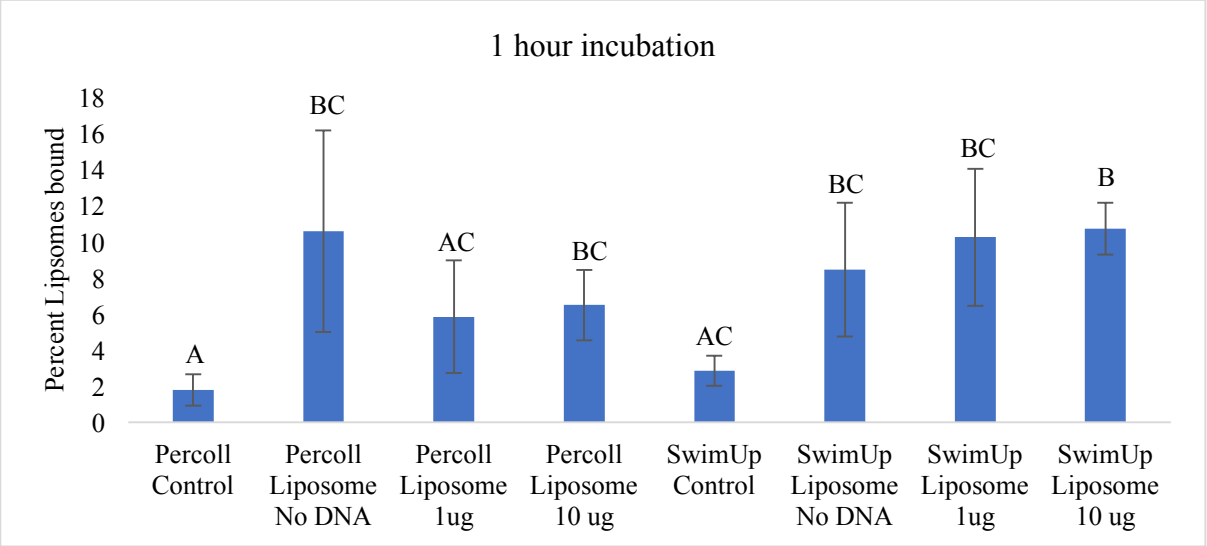
Table 6: Percent of liposomes bound to sperm

Table showing the average number of liposomes (± SE) bound to sperm for each group at time 0,1 and 3.

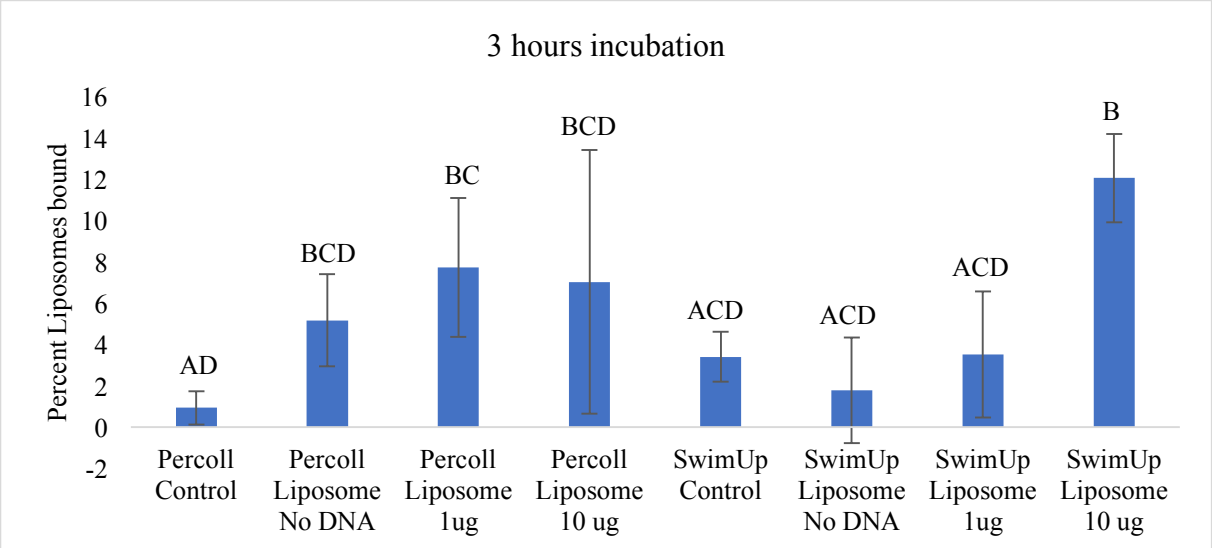
Time	Group	Average
0	Percoll Control	2.45 (0.98)
	Swim-up Control	2.46 (0.55)
1	Percoll Control	1.80 (0.87)
	Percoll Liposome 10 μg	6.51 (1.96)
	Percoll Liposome 1μg	5.85 (3.12)
	Percoll Liposome No DNA	10.59 (5.57)
	Swim-up Control	2.85 (0.83)
	Swim-up Liposome 10 μg	10.74 (1.43)
	Swim-up Liposome 1ug	10.26 (3.80)
	Swim-up Liposome No DNA	8.46 (3.70)
3	Percoll Control	0.95 (0.80)
	Percoll Liposome 10 μg	7.04 (6.37)
	Percoll Liposome 1ug	7.75 (3.36)
	Percoll Liposome No DNA	5.18 (2.22)
	Swim-up Control	3.42 (1.21)
	Swim-up Liposome 10 ug	12.06 (2.14)
	Swim-up Liposome μg	3.52 (3.05)
	Swim-up Liposome No DNA	1.80 (2.55)

Figure 18: Evaluation percent of liposomes bound to sperm based on group

Comparison of the percent of liposomes bound to sperm between different groups.



[ABC]Least square means (± SE) within each column with different superscripts differ (P<0.05).



[ABCD]Least square means (± SE) within each column with different superscripts differ (P<0.05).

SMGT

Embryos were made using SMGT, the sperm was incubated with five different treatments. After seven days (8 days post-fertilization), blastocysts were evaluated. The first set had 25 oocytes for each group and the treatments were repeated twice for a n of 50. The sperm in

this group was incubated for three hours before added to the well with the oocyte. The blastocyst rate was very low at 0%, 4% or 8% (Table 7). For the second replicate the sperm was also incubated for three hours, there were 30 oocytes per group this was repeated twice for an n of 60. The blastocyst rate was either 1.67%, 3.33% or 5% (Table 7). In the third replicate, sperm were only incubated with liposomes for one hour instead of three. For set three, oocytes used for group 1 and 2 came from dairy cows and group 3,4 and 5 were from beef cows. Group one n= 60 with a blastocyst rate of 8.33%, group two n=34 blastocyst rate 2.94%, group 3,4 and 5 n= 40 with a blastocyst rate of 2.5%, 20%, and 5% (Table 7). DNA was extracted from all blastocysts and none were positive for GFP.

Table 7: Summary of Blastocyst rate

The percent of blastocysts that were seen at day 8 after IVF for groups 1-5. This was repeated three times. For the first two replicates sperm were incubated with liposome-DNA complex for three hours. In the third replicate sperm was incubated with liposome-DNA complex for one hour.

Blastocysts %					
Incubation	**3 hours**		**1 hour**		
Group	**1**	**2**	**3**	**4**	**Average**
1	8.00%	3.33%	8.33%	18.00%	9.42%
2	4.00%	3.33%	2.94%	12.00%	5.57%
3	8.00%	1.67%	2.50%	15.00%	6.79%
4	0.00%	5.00%	20.00%	0.00%	6.25%
5	8.00%	3.33%	5.00%	5.00%	5.33%

3.5 Discussion

Sperm-mediated gene transfer was one of the first methods used to create transgenic animals. Since then microinjection has become the most commonly used method, but it requires greater skill and there are less reports of it being used in livestock species. Nuclear transfer is

more commonly reported but also requires more time and skill than SMGT. Although SMGT has been shown to work in a variety of species, there have been a small number of publications that successfully used this method in cattle. For this experiment, we used SMGT in combination with liposomes to deliver plasmid DNA containing a GFP selection marker. We were able to detect that plasmid DNA did bind to the sperm and determined the selection method that least effected sperm motility. However, we did not detect any GFP presence in the blastocysts, therefore were unable to transfer plasmid DNA.

This experiment used liposomes to assist in the delivery of plasmid DNA in combination with SMGT. The same method was used for gene transfer in pigs (3). This method used cationic lipids in combination with a neutral lipid, in this study they were able to create genetically modified pigs using SMGT. They found that 94% of 8 day embryos contained the GFP plasmid that they had transfected pigs with using artificial insemination AI and SMGT(3). In our experiment we used cryopreserved bull semen and selected sperm before incubating it with liposomes and DNA. This may explain the differences in results between these two studies.

Percoll and swim-up methods were used to select sperm. The motility of sperm was analyzed using CASA to determine the effects of liposomes and the different selection methods on the motility of the sperm. Based on the CASA results that looked at total, progressive and rapid motility; swim-up was the method that least effected sperm motility after being incubated with liposomes and 1 μg of DNA. To determine if plasmid DNA had successfully bound to the sperm, DNA was extracted from sperm and quantified using qPCR. SLIM was also used to look for the presence of liposomes on the head and tail of sperm. SLIM confirmed that there were liposomes on the sperm and qPCR did detect plasmid GFP DNA. This is the first report using

SLIM to measure liposomes and visualize their attachment to the sperm head and tail. Once the method that least effected sperm motility was determined, IVF was applied.

We were not successful in using SMGT to create edited embryos. The blastocyst rate was very low ranging from 0-8% after sperm was incubated with liposomes for three hours. The previous experiment had used fresh boar semen and AI, while in this study we used bovine semen that had been cryopreserved and selected, then performed IVF. Studies have shown that after selection, sperm motility begins to decrease and a significant difference in bull sperm motility can be seen between one and three hours (25). Originally sperm had been incubated for three hours, a second study was done that incubated sperm with liposome-DNA complex for one hour instead of three. However, we did not see a difference in sperm motility using CASA to analyze sperm that was incubated for one hour vs. three hours. Very low blastocyst rates (7.75%) were still seen when using sperm that was incubated with liposome mixture for one hour. Of the blastocysts that survived from both studies (41 out of 764 oocytes), DNA was extracted and then PCR was used to detect the presence of GFP. None of the blastocysts tested positive for GFP.

It is not surprising that we did not see any positive blastocysts. Over the years, few publications have shown SMGT to have high success rates, especially in cattle. One study saw that 22% of blastocysts showed the presence of the desired plasmid DNA using PCR, the blastocysts were made by incubating plasmid DNA with sperm for two hours and then performing IVF. In that experiment they analyzed 976 blastocysts, but the presence of the transgene could not be verified as proof of integration because the construction used was plasmid DNA and could have been remaining in the sample but not integrated into the blastocysts (9). Another study used AI instead of IVF; they inseminated 210 heifers, 41 of which gave birth and one tested positive for the inserted DNA (3.3%) (13). A higher percent of edited blastocysts were

seen when electroporation was applied to sperm. While 3.5% of embryos showed homologous recombination when sperm was incubated with DNA and no electroporation was applied, an increase to 55% of embryos had homologous recombination when electroporation was applied (26). Reagents such as FuGENE® 6 have also been used to insert DNA to embryos by incubating FuGENE® 6 with sperm for SMGT, but only 3.6% of blastocysts expressed GFP (27). Most of these studies had a blastocyst rate of at least 20% and examined between 30 and 100 blastocysts for each group. We were not able to obtain those kinds of numbers for this experiment which may be why we could not detect GFP. Previously mentioned studies all had very low success rates using SMGT in bovine. Lavitrano has had a much higher success rate in pigs, with 50-60% of piglets born contain the transgene (28). This was done by simply incubating sperm with DNA and then using AI (28). This may also explain why we did not see any GFP blastocysts, SMGT with fresh semen in pigs may have a higher efficiency than using frozen-thawed semen in cows. This study shows that when sperm is incubated with liposomes with ester linkages and DNA, DNA will bind to the sperm and that swim-up is the optimal method for selection before SMGT. We were unable to detect GFP in blastocysts that were made with IVF.

Originally one of the reasons that SMGT was used instead of nuclear transfer was because nuclear transfer requires a cell line with the desired edit. We had difficulty getting our fetal fibroblasts to survive, but from the few that did we were able to insert the desired mutation into on colony of Angus fetal fibroblasts. Although the survival rate was low, there is a possibility that nuclear transfer could be used in the future for gene transfer, if we can increase survival rate of fetal fibroblasts. We are also still unsure why the blastocyst rate was so low using

SMGT. The experiment may need to be repeated multiple times in order to get enough blastocysts to survive and detect gene transfer using liposomes and SMGT.

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General Discussion

Gene editing has been around for many years and now it can be done with greater precision and ease because of the CRISPR/Cas9 system. Currently gene editing has been applied to the study of a variety of cell lines and mice. It has been shown that CRISPRs can create edits in the pig genome as well as other livestock species (1). Two studies have shown successful editing of the cattle genome in bovine fetal fibroblasts, which were used to make embryos via nuclear transfer (2,3). No cattle embryos edited using the CRISPR/Cas9 system have made it to the live birth stage at this point, to our knowledge.

The main goal of this experiment was to determine if the CRISPR/Cas9 system could be used to make a single nucleotide change in the bovine genome. For this project, the α-LA gene sequence in the cattle genome was targeted because of the role it plays in milk production (4). The single nucleotide change in this gene is found in Holstein cows and is associated with increased milk production. α-LA combines with β-1,4 galactosyltransferase to produce lactose, a major sugar in milk. Lactose also functions as an osmole-regulator, which means that as lactose production increases the volume of milk also increases (5).

MAC-T cells were chosen as the cell line of interest to provide a proof of principle for introducing this single nucleotide change. MAC-T cells are immortal cells that were isolated from the Holstein mammary gland and can be induced to produce milk proteins. Fetal fibroblasts were also transfected to see if they could be used at a later point for nuclear transfer. Using CRISPR/Cas9 plasmid DNA with an inserted gRNA and a GFP selection marker, we were able to create a double strand break in the desired location of the α-LA gene sequence. MAC-T cells were transfected using FuGENE® 6 and then sorted into a 96-well plate. MAC-T cells were first transfected with FuGENE® 6 with pIRES2-EGFP, resulting in 30% fluorescence of surviving

cells. This was lower than want had been seen in other cells lines, therefore we optimized transfection protocols with Lipofectamine® 2000 and electroporation to see if we could increase the proportion of cells that fluoresced. Lipofectamine® 2000 resulted in a wide range in fluorescence (20-40%), and a large amount of cell death. Lipofectamine® 2000 proved to be too toxic for MAC-T cells. Electroporation was also tested to increase transfection efficiency (30-40%), this method also resulted in massive cell death. Based on these results, FuGENE® 6 was used for the remaining experiments because it was the least toxic reagent for transfection MAC-T cells.

Cells were then analyzed using enzyme digestion with BpuEI. Originally surveyor assay was used to determine if the CRISPR/Cas9 plasmid was creating a break near the desired mutation. We continuously saw a double band in our control group, signifying that a mismatch in the sequence was already present. This led us to try a different detection method. By using enzyme detection with BpuEI, we discovered that MAC-T cells were heterozygous for the α-LA (+15) mutation and had a guanine and an adenine. Which explained why the control for surveyor assay resulted in two bands on an agarose gel.

After verifying that the CRISPR was cutting at the desired location the CRISPR/Cas9 plasmid DNA was delivered with ssODN that was 90 bp long with a single nucleotide change with an adenine at the (+15) position in the 5' flanking region. In the MAC-T cells we were not able to make an insertion but one of the colonies had a twenty-nucleotide deletion. This may be useful in future studies, the deletion included the transcription and translation start point of α-LA, which may change the synthesis of this milk protein in cultured MAC-T cells. Because both survival rate and cutting efficiency were low in MAC-T cells, this experiment needs to be repeated with more cells in order to see homology directed repair. HDR naturally occurs at a

lower frequency than NHEJ, therefore with low amounts of NHEJ, large number of cells would need to be evaluated.

MAC-T cells have issues that make them a difficult cell line to work with for these studies. The cells were grown in DMEM with 10% fetal bovine serum (FBS), 1 ml/L Pen/Strep, 50mg/L gentamycin and 1 mL/L Amphotericin B. Although there is specified growth media for MAC-T cells and milk component induction media, we found that cells grew proficiently in the media stated previously (6). Originally MAC-T cells were transfected with pSpCas9(BB)-2A-Puro. The problem with this plasmid was that cells would continuously die when puromycin was added. Using pIRES2-EGFP we confirmed that MAC-T cells were taking up plasmid DNA, therefore some should have been resistant to puromycin when transfected with pSpCas9(BB)-2A-Puro. A kill curve was performed multiple times to determine the optimal amount of puromycin for selection. MAC-T cells continued to show massive cell death and could take up to a month to recover after two days of puromycin selection. We then tried using pSpCas9(BB)-2A-GFP which allowed us to sort cells into 96-well plates using FACS. We saw a range of one to thirty-six (36) colonies that would form after a sort. Of those colonies that survived 0 to 21% had a double strand break that resulted in indels. With such a low number of indels it is not surprising that we did not see a single nucleotide change using a ssODN, because HDR occurs at an even lower frequency than NHEJ.

There are multiple factors that may contribute to the low transfection and survival rate of MAC-T cells. It could be because of the cell line, the reagent being used to transfect cells, or the way in which the CRISPR/Cas9 system was delivered. One study recently looked at 60 different transfection reagents used to deliver Cas9 protein in a variety of cell lines. They saw that Lipofectamine™ CRISPRMAX™ worked in most cell lines but in MCF-7 cells (human

mammary gland) they saw very low percent indels formed with all transfection methods. Lipofectamine™ CRISPRMAX™ only resulted in 8% indel formation in MCF-7 cells, and other transfection methods were even lower (7). These results lead me to question whether the difficulty to attain successful transfections rates is a characteristic of mammary cell lines.

Angus fetal fibroblasts were also transfected with pSpCas9(BB)-2A-GFP and then sorted into a 96-well plate. The fetal fibroblasts did not carry the mutation and PCR and enzyme digest resulted in a single band at 420 bp. To detect the cutting efficiency of CRISPR/Cas9 surveyor assay was used. These cells had an even lower survival rate than MAC-T cells at ~10%, but the cutting efficiency was much higher. Since NHEJ was higher in these cells we were able to insert the ssODN and create the desired insertion at α-LA (+15), but it was only seen in one of the eight surviving colonies. These cells had a very low survival rate. If this experiment was repeated greater amounts of insertion may be seen.

Angus fetal fibroblasts are a primary cell line while MAC-T cells are an immortal cell line. This may be why we see such a difference in survival and editing rates. With the fetal fibroblasts, we only saw 1-6 colonies form in a 96-well plate. However, of the cells that survived ~50% of them showed indels and we were able to make a single nucleotide change using a ssODN. Fetal fibroblasts may have had a low survival rate when they were sorted because they require specific media with FGF, and we had some issues with our incubators and our media. Another potential reason is that cells in culture have a higher survival rate when there are multiple cells plated together. After FACS sorting, 5 cells were seeded per well. If this number was increased, there would be a risk that more than one cell may attach but it could lead to a higher survival rate. In order to get a more accurate reading of HDR and NHEJ, this experiment would need to be repeated in an effort to gain a greater proportion of colony survival. Instead of

sorting the cells into a 96-well plate, the fluorescent cells could be pooled into a tube. From the pooled samples DNA would be extracted then amplified by PCR, to clone the PCR fragments into plasmid DNA, followed by sequencing of individual plasmid DNA. While this would not allow selection of positive cells it would provide the percent of NHEJ and HDR that is occurring.

Once the CRISPR/Cas9 system was verified in cells lines, the next step was to create an edited embryo. In mice, the common method of gene transfer is through microinjection. This method is less efficient and requires greater skill to perform in cattle and other livestock species. Nuclear transfer is a more commonly used method of gene transfer but because of our low survival rate of fetal fibroblasts we chose to use sperm mediated gene transfer (SMGT). SMGT was used in combination with liposomes to increase the efficiency of gene delivery. Percoll and swim-up were two selection methods that were analyzed along with 1 μg and 10 μg of DNA. Then the sperm were analyzed using CASA to look at the effects of the liposome and liposome DNA complex on sperm motility. Sperm was originally incubated with the liposome complex for three hours after sperm had been selected by Percoll gradient or swim-up. From this preliminary experiment, we determined that sperm selection using swim-up with 1 μg of DNA has the least effect on sperm motility. The ability of the liposome-DNA complex to bind to the sperm was confirmed by qPCR and SLIM.

IVF was then performed with sperm incubated with liposomes. After seven days (8 days post-fertilization) embryos were analyzed and we saw a blastocyst rate of ~6%. Of the blastocysts that survived none were positive for GFP plasmid DNA. We believed that this low blastocyst rate may have been a result of waiting too long between sperm selection and IVF that the sperm motility may have decreased. In order to increase blastocyst rate the sperm were incubated with liposomes for one hour instead of three. However, we did not see a difference in

sperm motility or blastocyst rate when sperm was incubated for 1 hour vs. 3 hours. Again, none of the blastocysts that survived were positive for GFP plasmid DNA.

Of 974 oocytes that were evaluated after 8 days for five different groups, only 64 were blastocysts. On average the percentage of blastocyst development is between 40% and 50% (8), and we saw between 0% and 20%. This low percentage is not due to the presence of liposomes because even in the control IVF group there was a low amount blastocyst development. These rates may be due to the quality of the bull semen that was used, higher quality sperm may result in an increased blastocyst rate. Since the rate of blastocyst produces was so low it is not surprising that we did not see any that had taken up the plasmid DNA. SMGT has a low efficiency especially in cattle, the liposomes that were used in this experiment were previously shown to work well in pigs using fresh semen (9-11). Unfortunately, this system did not transfer well to cattle using frozen bull semen. One study compared fresh bull semen to frozen and saw that frozen took up more exogenous DNA than fresh (12). However, they did not use this sperm for SMGT and did not have any blastocysts to evaluate. SMGT may be an efficient method for transferring exogenous DNA into pigs, but there has not been wide success in cattle. Pigs also have a larger litter size than cows and a shorter gestation period. It is more difficult to do genetic engineering in cattle because it requires a longer amount of time and more money. In order to create an edited embryo, it may be more efficient to increase the survival rate of fetal fibroblasts and use nuclear transfer, since we were able to confirm that fetal fibroblasts can be edited using CRISPRs.

While we did not achieve our desired end goal in this experiment we were able to evaluate the use of the new imaging technique SLIM. SLIM was designed at the Beckman Institute in the Dr. Gabriel Popescu's lab. Using this system, we can take clear images of sperm

and see areas of greater density on the head and tail of the sperm, which we believe are liposomes. This is the first time this method was used and the system is still being modified. The first time we evaluated slides I was unable to analyze most of them because the areas which contained sperm were too dense and had a lot of debris. For the next few slides, samples were spread out before evaluation. By using the jet color map on imageJ, sperm density was evaluated. The areas of greater density were red; when looking for quality sperm images I chose sperm with intact head and tail, and that were not overlapping. Using SLIM to evaluate sperm images with liposomes still needs to be modified. In order to better evaluate this method I think we need a control that was incubated with DNA and no liposomes. Or a way to stain the liposomes so we can confirm that the regions of higher density are liposomes.

CRISPRs have provided a new method to genetically modify animals and can create knock-outs and knock-ins in specifically targeted areas of the genome with higher precision. With this advancement in technology we need to also improve our gene transfer methods. The combination of these techniques could lead to advancement in medical models, increased animal production, and understanding of the functions of different genes.

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Appendix A

Designing CRISPR Plasmid

Objective: Insert guide RNA into CRISPR plasmid that will guide the plasmid to create a double strand break in DNA.

Transformation Protocol

Thaw Stellar Competent Cells in an ice bath just before use. After thawing, mix gently to ensure even distribution, and then move 50 µl of competent cells into a 14-mL round-bottom tube. Do not vortex. Add no more than 5 ng of DNA for transformation. Place tubes on ice for 30 min. Heat shock the cells for exactly 45 sec at 42°C. Place tubes on ice for 1–5 min. Add SOC medium to bring the final volume to 500 µl. SOC medium should be warmed to 37°C before using. Incubate by shaking (160–225 rpm) for 1 hr at 37°C. Plate an appropriate amount of culture on selective medium.

Inoculating an Overnight Liquid Culture of Addgene Plasmids

Everything should be done in a hood or by a flame. Follow addgene protocol after receiving plasmid. Add 5 mL of LB into 15 mL tube then add 50 µl of 10mg/mL amp stock. Use toothpick, pipet tip, or loop to pick a colony from the plate and shake into LB+amp, then shake overnight at 37° C for 12-18 hours.

Inoculating an Overnight Glycerol Stock

Everything should be done in a hood or by a flame. Plasmids are frozen at -80 ° C. Use 5 mL of LB, put into 15 mL tube, add 50 µl of 10mg/mL amp stock. Use toothpick, pipet tip, or loop; scratch top of stock LB+amp then shake overnight at 37° C for 12-18 hours.

Extract DNA from Plasmid

Harvest the bacterial culture by moving culture into 15 mL tube and then centrifugation at 4000 rpm for 8 minutes at room temperature. Decant the supernatant and remove all remaining medium. Use GeneJET Plasmid miniprep kit (Thermo Fisher Scientific, Waltham, MA) to extract DNA. Add resuspension solution, lysis solution and neutralization solution according to protocol according to the kit. Bind the DNA in a column, wash the column, and then elute the purified DNA in 50 µl of elution buffer. Send DNA to sequencing at UIUC Core Sequencing Facility (Urbana, IL) with U6 forward primer to detect if the sgRNA had successfully inserted into the plasmids.

Cloning sgRNA into Cas9

Prep of sgRNA oligo inserts. Order sgRNA oligos from IDT. Add 1 µl of sgRNA top (100 µM), 1 µl of sgRNA bottom (100 µM), 1 µl of T4 ligation buffer (10×), 1 µl of T4 PNK, and 6 µl of ddH2O for a total of 10 µl. Amplify on thermocycler at: 37° C 30 min, 95° C for 5 min then ramp down to 25° C at 5 min. Dilute oligos 1:200 by adding 1 µl to 199 µl.

Cloning the sgRNA Oligos into psCas9

Add together 100 ng of pSpCas9(BB), 2 µl of Diluted oligo duplex, 2 µl of Tango buffer (Thermo Fisher Scientific, Waltham, MA) (10×), 1 µl of DTT (10 mM), 1 µl of ATP (10mM), 1 µl of FastDigest BbsI, 0.5 µl of T7 ligase, total amount of 20 µl with ddH2O. Incubate for six cycles at 37° C for 5 min, 21° C for 5 min.

Ligation Reaction with Plasmid Safe Endonuclease

Add together 11 µl of Ligation reaction, 1.5 µl of PlasmidSafe buffer (Thermo Fisher Scientific, Waltham, MA) (10x), 1.5 µl of ATP (10 mM), and 1 µl of PlasmidSafe exonuclease for a total of 15 µl. Then incubate at 37° C for 30 min 70° C for 5-10 min. Transformation (follow protocol above). Add 2µlfrom the reaction to the competent cells for 15 minutes. Plate onto LB + amp resistant plates. After 24 hours pick colonies and grow them in 3 mL with 30µlof 10mg/mL ampicillin. Extract DNA using GeneJet Plasmid miniprep kit from Thermo Scientific (Thermo Fisher Scientific, Waltham, MA).

Growing Competent Cells

Take 5mL from original stock of cells and inoculate in 5 mL of LB and let it grow for 7-10 hours at 37° C on a shaker. Bacteria should grow exponentially. Calculate ODi needed for x amount of hours overnight.

$OD_F = OD_i * 2^{((t-L)/t_d)}$

t= incubation time L= lac phase 3.5 hours T_d= 4.5

ex. $C_1V_1=C_2V_2$; C1 comes from measuring OD 0.05 * 200mL = 3mL* V_2

V_2= .3; add .3 mL of V_2 to 200mL of V_1

Grow cells overnight in SOB, Measure the OD the next morning. Once OD is 0.4-0.6 transfer the cells into 50 mL tubes (200mL=4 tubes). Place tubes on ice for 5 minutes then centrifuge tubes for 10 minutes at 4000 rpm at 4°C. Bring samples to a cold room and dump the supernatant and re-suspend the cell pellet with wash buffer. The buffer is at 2x and it need to be at 1x. Use a 1:1 ratio. In a separate tube pipette in 10 mL of wash and 10 mL of competent wash then add 5 mL to each 50 mL tube in order to create 1:1 dilution. Vortex the samples then combine 2 of the tubes so there are now 2 tubes instead of 4. Centrifuge tubes for 10 minutes at 4000 rpm at 4°C. In a separate tube combine 10 mL of wash and 10 mL of competent wash. Mix well. Add 10 mL of mixture to each tube. Vortex tubes and then combine them into one tube. Pipette cells into tubes that are on dry ice and freeze in -80°C. When doing this for the first time check the OD every hour during the 10 hours of inoculation. Keep competent cells at -80. For the small culture use LB, for the large culture use SOB so it is the same as the mix and go kit.

Appendix B

MAC-T cells and Fetal Fibroblasts Transfection

Objective: Insert desired plasmid into cell lines.

Thawing Cells

Obtain cells in a cryovial from the liquid nitrogen tanks and immediately place in water bath. Thaw cells quickly (<1miniute) in 37˚C water bath until there is just a small bit of ice left in vial. Transfer vial into laminar flow hood and wipe down outside of vial with 70% ethanol. Rinse cells by combining 5mL of DMEM with contents of cryo-vial (1mL). Add DMEM dropwise to the cells. Centrifuge at 1000rpm for 5 minutes at room temperature. Decant supernatant into waste flask making sure to not lose the cell pellet. Re-suspend in 10 mL DMEM 10% FBS. For fetal fibroblasts use 10% FBS, 0.01µg/mL basic fibroblast growth factor (bFGF), 1 mL/L Pen/Strep and 1 mL/L Amphotericin B. Place media and cells into 75cm² flask add another 5 mL of media to the flask for a total volume of 15mL. Place flask in incubator with 5% CO_2 in air; 100% humidity at 39ºC.

Freezing Cells

Freezing media is composed of 60% DMEM, 20% Dimethyl Sulfoxide (DMSO) (Thermo Fisher Scientific, Waltham, MA), 20% FBS. Freeze cells when 80% confluent, trypsinize the cells and spin down at 1000 rpm for 5 minutes. Resuspend cells in DMEM and find concentration. Freeze cells at concentration of $1x10^6$ cells per vial in a 1 mL volume, add 1 mL of freezing medium to the vial. Freeze at -80ºC for at least one day and after place in liquid nitrogen.

Passaging Cells

Remove media in flask either by pouring off gently into waste flask or by pipetting media off. Pipette 5 mL of PBS into flask to rinse the cells, remove PBS. Pipette 3 mL of trypsin into 75cm² flask making sure to cover the entire bottom area of the flask. Place flask in incubator, wait about 8-15 minutes for MAC-T cells and 5 minutes for Fetal fibroblasts. Then gently tap the flask to remove remaining attached cells from the flask. Look at cells under the microscope to see if detachment has occurred; trypsin should not be left on the cells for longer than 3 minutes. Add equal amounts of DMEM to the flask after cells have detached. Gently wash remaining cells off the flask by pipetting the DMEM/trypsin mixture up and down. Remove all the DMEM/trypsin and put into 15mL tube. Centrifuge 1000 rpm, remove supernatant and re-suspend the pellet in DMEM or specified medium. Count the cells and plate to the concentration designed for appropriate cell density based on the size flask you are using. (5,000 -10,000 cells per cm^2). Add DMEM to equal a total of 15mL in the flask.

MAC-T Cells Transfection

Cultivate MAC-T cells in a 75 cm^2 flasks in DMEM with 10% fetal bovine serum (FBS), 1 mL/L Pen/Strep, 50mg/L gentamycin and 1 mL/L Amphotericin B. Change media every other day. Plate 150,000 cells the day before transfections in a 6-well plate with DMEM (high glucose, Sigma-Aldrich, St. Louis, MO), 10% FBS and no antibiotics. Once they reach 70% confluency transfect MAC-Ts with FuGENE® 6 Transfection Reagent (Promega, Madison, WI). Add 2 μg of DNA to 100 μl of opti-MEM (Thermo Fisher Scientific, Waltham, MA), then add 6 μl of FuGENE® 6 Transfection Reagent to the Opti-MEM and leave at room temperature for 15 minutes. When MAC-T cells are transfected with ssODN, use 10 or 30 pmol. The mixture is then

added dropwise to the well and put in the incubator. After 24 hours, image the cells using a florescent microscope Olympus IX71 to determine the percent transfected.

Angus Fetal Fibroblast Transfection

The Angus fetal fibroblasts, gifted from Dr. Jonathan Beever's lab (Urbana, IL), were grown in DMEM/F10 media (Cell Media Facility, Urbana, IL) supplemented with 10% FBS, 0.01µg/mL basic fibroblast growth factor (bFGF) (Sigma-Aldrich, St. Louis, MO), 1 mL/L Pen/Strep and 1 mL/L Amphotericin B. Carry out transfectionas stated previously using FuGENE® 6 Transfection Reagent.

Cell Selection

24 hours after transfection bring cells to ERML to be flow sorted. Bring 96-well plates with desired 200 mL of media, and a control cell line that has not been transfected. After cells are sorted place them in an incubator. Check after three days, wait a week before changing the media. Once cells are confluent trypsonize them with 50µl trypsin, wait 10 minutes (or until detached) add 50µl of media pipette up and down. Moved to 24-well plate, each well should have 400µlof media. Wait till cells are confluent in the 24-well plate to extract DNA. Add 150µlof trypsin and wait 10 minutes. Move to 1.5 mL tube and spin tube down at 1000 rpm for 5 min. Carefully remove media then ass 50µlof QuickExtract™ and place on thermocycler at 65°C for two hours and finish with 98C for 10 minutes. DNA was amplified by PCR and then analyzed using surveyor assay or enzyme digestion. Once 6-well plate is confluent freeze and save cells.

PCR Mutation Detection

The α-LA sequence was analyzed by conventional PCR using α-LA (+15) forward primer (TGGACCCTT TGTGCATTTTCT) and α-LA(+15) reverse primer (TGGGTGGCATGGAATAGGAT). Combine these with JumpStart™ Taq DNA Polymerase (Sigma-Aldrich, St. Louis, MO) run on cycling parameters: 30 sec at 94°C, 45 sec at 60°C, 45 sec at 72 °C, second two 30 times, 10 min 72°C, and 5 min 4°C. Digest the PCR product with restriction enzyme BpuEI (New England BioLabs, Ipswich, MA) at 37°C for one hour. Cuts are analyzed on a 2% agarose gel. MAC-T cells are heterozygous for the mutation at the (+15) site, they have both adenine and guanine. Non-edited cells show two bands, one at 420 bp and one at 370 bp, cells that are edited have a frameshift or deletion and result in one band at 420. Cells that showed two bands should be discarded, while cells that have one band will be transferred to a 6-well plate and frozen -80 in freezing media 80% DMEM with 10% FBS, 10% DMSO (Thermo Fisher Scientific, Waltham, MA), and 10% FBS.

Electroporation

Electroporate MAC-T cells using Gene Pulser® II Electroporation System (Bio-Rad, Hercules, CA) with an attached capacitance extender. Set voltage capacitance at 950 μF, with four different voltages, 250V, 300V, 350V, and 400V. Suspend 5 million or $2.5X10^6$ MAC-T cells in 400 μl of Opti-MEM and place in a tube with 10 μg of plasmid GFP DNA, then transfer to a cuvette and load onto the Gene Pulser. Cells were then placed in a 6-well plate, after 24 hours media was changed and cells were imaged to determine the efficiency. Optimize different voltages; 100V, 150V, 200V, and 220V in 400 μl of Opti-MEM with 10 μg of DNA. Then optimize capacitance. $2.5X10^6$ MAC-T cells were put in tubes with 400 μl of Opti-MEM and 10

μg of DNA. Pule at a voltage of 220V or 150V and a capacitance of 350 μF, 500 μF, or 950 μF. After the medium has been changed do antibiotic selection with 0.5 μg/mL of puromycin for two days. Allow cells to recover and extract DNA.

Lipofectamine

Plate 200,000 24 hours before transfecting cells in a 6-well plate with DMEM, and 10% FBS. Add varying amounts of Lipofectamine® 2000 (Thermo Fisher Scientific, Waltham, MA) and DNA to Opti-MEM separately and then combine the two. After 20 minutes add the mixture to the cells and place back in the incubator. Lipofectamine® 2000 can be added at 6, 7, 7.5,8, 9, and 10 μl, with 2.5 or 3 μg of DNA, add to Opti-MEM. After 6 hours remove the media and replace with DMEM, 10% FBS, 1 mL/L Pen/Strep, gentamycin 50mg/L and 1 mL/L Amphotericin B. Image the cells after 24 hours to look at GFP expression. Change media and add 0.5 μg/mL of puromycin for selection. Do this for two days then allow cells to grow back and extract DNA.

Appendix C

Sperm-Mediated Gene Transfer using Liposomes

Objective: Bind DNA to sperm head in order to transfer DNA to make an edited embryo.

Liposome Production

The cationic lipid, 3-(trimethyl ammonium iodide) 1,2 dimystryl-propanediate (TAID) was synthesized by Russel in (3) and can be obtained from lab. The neutral lipid, L-α Dioleoyl phosphatidyl-ethanolamine (DOPE) is purchased from sigma (Sigma-Aldrich, St. Louis, MO) and can be used without further purification. Reconstitute DOPE in a 9:1 ration of CHCl3:MeOH , once mixed, dissolve TAID and DOPE in an organic solvent (CHCl3) at a molar ratio of 2:1 in a round bottom flask. Use a rotary evaporator to concentrate the lipid at the bottom of the flask at 40-50°C. Add one quarter of the final volume of TALP to the round bottom flask. Use a cell scraper to scrape the layer of lipids from the glass surface, then pipette the sample up and down for one minute follow with a minute of vortexing. Place the flask in an incubator at 37°C for 15 minutes at 5% CO_2. Add 50 µl of the liposome TALP mix to tubes containing zero, one, or 10 µg of DNA. Incubate the liposome/DNA complexes at 37°C for 15 minutes at 5% CO_2. Add collected sperm to medium alone (control) or medium containing liposome/ DNA complexes. Add 50 µl to 150 µl of TALP and sperm. The final concentration of the solution should be 150 µM lipids. Place the mixture at 37°C in 5% CO_2 for three hours.

Percoll Gradient

Percoll discontinuous (45% to 80%) gradient is prepared by combining Sperm-TALP and ISO-Percoll. Sperm-TALP- basis- medium, is added to pyruvic acid and gentamycin sulfate. ISO-

Percoll is made by combining sodium bicarbonate with Earle's Balanced Salt Solution (EBSS) (Thermo Fisher Scientific, Waltham, MA) and vortexed in a tube. This is then combined with Percoll® (Sigma-Aldrich, St. Louis, MO), the pH is adjusted to 7.4 and then the solution should be filtered through a .22 μm filter. Combine ISO-Percoll and Sperm-TALP to create 45% and 80% mixture of Percoll. First ass 80% Percoll to the tube then slowly add 45% Percoll as a second layer. Pool frozen-thawed bull semen from liquid nitrogen tanks and immediately place in warm water at 37°C for 40 seconds. Slowly pipette semen down the side of the tube to create a third layer on top of the 45% ISO-Percoll, then centrifuge at 460 x g for 25 minutes. Discarded the supernatant and wash the pellet in Sperm-TALP, then centrifuge at 250 X g for 10 minutes. Repeat this three times, removing the supernatant and suspending the pellet in Sperm-TALP and centrifuging it at 170 x g for 10 minutes. Remove the supernatant and add 50 μl of Sperm-TALP to the pellet. Evaluate using CASA.

Swim-up

Thaw semen samples and layer them carefully under 1 mL of equilibrated sperm-TALP (Tyrode's albumin lactate pyruvate) medium with 6 mg of BSA per mL in a centrifuge tube. After loading, place the tube in the incubator at 39 °C for 1 hour. After incubation, collect 400 μL of the upper fraction of TALP (containing the selected sperm), place in a tube and centrifuge for 10 min at 160 x g (116).

Sperm Genomic DNA Extraction

Thaw frozen samples at room temperature. Spin down at 15,600 x g for five minutes and remove supernatant. Add 500 μl of 70% ethanol to the pellet and centrifuged again at 15,600 x g for five

minutes. Lyse cells by adding 500 µl of lysis buffer, composed of 2 mL of 5 M NaCl, 1 mL of 1M tris, 2.5 mL of 1M EDTA (Thermo Fisher Scientific, Waltham, MA), 5 mL 10% SDS, and 89.5 mL dd H_2O for a final volume of 100 mL. Then add 2.5 µl of 0.5% Triton® X-100, 21 µl of 1M dithiothretol (DTT), and 40 µl of 10 mg/µl of proteinase K (Invitrogen- Thermo Fisher Scientific, Waltham, MA) to each sample. Vortex and incubated at 50°C overnight on a shaker with moderate shaking. The following day spin samples for 10 minutes at 15,600 x g and transfer supernatant to a new 1.5 mL tube. Precipitate the DNA by, adding 1 µl of 20 mg/mL glycogen and 1/10 volume of 3M NaAc the supernatant. Add two volumes of ice-cold absolute ethanol and placed in -80°C for 1-2 hours. Pellet DNA by centrifuging the sample for 20 minutes at 15,600 x g. Remove supernatant being careful not to disturb the pellet, wash pellet with 500 µl of 75% ethanol and centrifuged for 10 minutes at 15,600 x g. Remove ethanol and leave at room temperature until the ethanol has evaporated. Dissolve DNA in 30 µl of TE buffer, which is composed of 10 mL of 1 M TRIS pH 8.0, 1 mL of 1M EDTA, and bring to a final volume of 1000 mL with ddH_2O. Leave DNA at 4°C overnight, measured the concentration the next day using a NanoDrop. Store DNA at -20°C.

Quantitative Real-time PCR (qRT-PCR) analysis

Use SYBR® Select Master Mix for CFX (Thermo Fisher Scientific, Waltham, MA) to run qPCR. Add 6 µl of SYBR select mix, 0.4 µl of forward and reverse primer, 4 µl of gDNA and 0.2 µl of ddH2O for a total volume of 10 µl. Use thermo cycling parameters; stage 1: 50°C for 2 min, stage 2: 95°C for 10 min, stage 3: 95°C for 15 sec then 65°C for 1 min repeated 40 times, stage 4: 95°C for 15 sec then 60°C for 15 sec. Do three replicates for quantification of the target gene. YWHAZ can be used as a housekeeping gene for bovine sperm and GFP can be used as a

primer to amplify GFP plasmid. Primers to detect eGFP are (CATGGTCCTGCTGGAGTTCGTG) and (CGTCGCCGTCCAGCTCGACCAG). The control primes for YWHAZ used are F (GCATCCCACAGACTATTTCC) and reverse primer (GCAAAGACAATGACAGACCA).

Embryo Production

Liposomes are prepared as stated previously, 1 μg of DNA is added to 50 μl of liposome TALP. Select and then count sperm and divided into one million sperm per 4 μl of TALP. Take 1 μl form the 50 μl liposome DNA mixture and then add to 4 μl of sperm TALP. Incubate sperm and liposome mixture at 37°C in 5% CO_2 for one to three hours. After one or three hours, remove sperm from the incubator. Add treated sperm to well containing 30-50 oocytes. Oocytes should be in IVF medium, Tyrode's modified medium (117) without glucose and bovine serum albumin (BSA), and supplemented with 5.3 SI/mL heparin, 30 μM penicillamine, 15 μM hypotaurine, 1 μM epinephrine and 1% of BS. Co-incubate gametes for 20 h at 39°C, in 5% CO_2 in air, after 20 h vortex zygotes for 2 min and pipette to remove cumulus cells in HEPES-TCM with 5% BS. Wash twice in the same medium and transfer 30–50 zygotes per well into 400 μl Synthetic Oviduct Fluid (SOF) medium (118), supplemented with 30 μl/mL essential amino acids, 10 μl/mL non-essential amino acids, 0.34 mM tri-sodium citrate, 2.77 mM myo-inositol and 5% BS. Incubate zygotes in a humidified mixture of 5% CO_2, 7% $O_2$and 88% N_2 in air at the temperature of 39°C.

Embryo Analysis

Extract genomic DNA by adding 20 µl of lysis buffer containing 15 mM Tris–HCl pH 8.9, 50 mM KCl, 2.5 mM MgCl2, 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) and 150 µg/mL proteinase K (Sigma-Aldrich, St. Louis, MO). Incubate tubes at 55°C for 1 h and then at 90°C for 10 min to inactivate proteinase K. Evaluate DNA using PCR with JumpStart™ REDTaq® DNA Polymerase (Sigma-Aldrich, St. Louis, MO). Use primers for GFP and control primers btRep-137 C1 and btRep-137 C2. The control primers are btRep-137 C1 (TATTTTCGGAACGCGGGAGAGAAGAG) and btRep-137 C2 (TATTTTTGATTCCCTCCGTGCGGCGCTTA) which result in 450 bp, the primers for GFP are forward primer (ACCTACGGCGTGCAGTGCTT) and reverse primer (AGGACCATGTGATCGCGCTT) resulting in 467 bp product. The GFP primers amplify the GFP sequence in both pIRES2-EGFP and pSpCas9(BB)-2A-GFP plasmids. PCR cycling parameters are: 1 min at 94°C, 30 sec at 94°C, 30 sec at 60 °C, 1 min at 72 °C, go to step 2 and repeat 30 times, 1 min 72°C, and 5 min 4°C. Run samples on a 2% agarose gel.